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Regulation of staphylococcal enterotoxin biosynthesis

Final Report

John J. Iandolo

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) <p>The genetics of enterotoxigenesis in <u>S. aureus</u> is being pursued. An element of Enterotoxin B (SEB) has been shown to exist as both a chromosomal and plasmid gene in methicillin resistant strains. Most of these exhibit chromosomal genes although 37.5% possess a 1.15 Mdal plasmid which is essential for SEB expression. Either genetic arrangement appears genetically linked to methicillin resistance although actual physical linkage has been ruled out. The association seems to be mediated by a tetracycline resistance plasmid.</p>																	

1. Summary:

The objectives of this research project were to genetically and biochemically extend our understanding of the regulation of the enterotoxins produced by the staphylococci. In particular, we needed 1) to know why enterotoxin B (SEB) existed as a plasmid gene in some strains and a chromosomal gene in other strains, 2) why either genetic conformation of SEB was intimately associated with both methicillin (*mec*) and tetracycline (*tc*) resistance for establishment and transfer, 3) how tetracycline plasmids (and possibly other small plasmids) strongly influenced the expression of the SEB gene, *entB*, 4) to extent genetic studies to include the 5 described serotypes of staphylococcal enterotoxin. The methods of investigation relied on transformational and transductional analysis to obtain genetic data; on plasmid characterization by restriction mapping and cloning and by *in vitro* protein synthesis of enterotoxin genes.

A summary of results obtained thusfar is as follows:

- 1) Plasmid DNA analysis of 16 *mec*^r SEB⁺ isolates showed that the *entB* plasmid was present in only 6 strains (37.5%). All of the other isolates contained typical penicillinase plasmids and smaller plasmids responsible for tetracycline or chloramphenicol resistance.
- 2) Genetic analysis of two strains (*S. aureus* DU-4916 and 592) which contain the 1.15×10^6 dalton *entB* plasmid confirmed that the toxin gene was extrachromosomal while similar studies of isolates which lack the *entB* plasmid demonstrated that the gene was chromosomal.
- 3) Transduction and transformation of *mec*^r to sensitive strains did not reveal linkage of *mec* and *entB*. However, both genes could be cotransduced at low frequencies with a plasmid responsible for resistance to tetracycline. We have proposed that the tetracycline resistance plasmid may act as an intermediate vector in the establishment of the *mec*^r SEB⁺ phenotype. The data presented in the enclosed manuscript (appendix 1) suggests that although these 3 genetic elements are not linked they are capable of a transient interaction.
- 4) Transduction of *entB* into a recombination deficient host showed that only plasmid *entB* genes could be so transferred. Chromosomal *entB* was not established. This finding suggests that chromosomal *entB* does not possess the ability to translocate. However, because of the inability to directly select for *entB* further evaluation of transposability is needed. These data are presented in the manuscript designated appendix 2.
- 5) We have also extended our studies on the genetics of enterotoxin synthesis to enterotoxin SEC₂. We have examined the plasmid DNA profile of 3 SEC₂ isolates and have found that these strains contain only a large plasmid (17.5×10^6 daltons) which is responsible for resistance to cadmium. In relationship to patterns already established we presume this is tentative evidence of a chromosomal locus for SEC₂.

6) Transformation of pSN2 into the Bacillus subtilis genetic background failed to induce the production of enterotoxin B. Further minicell and in vitro translation data show that pSN2 does not carry the entB structural gene. Nevertheless, the plasmid is essential for enterotoxigenesis. In fact, the regulatory functions provided by pSN2 are also contained in the chromosome of those S. aureus strains that do not harbor this plasmid.



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FOREWORD:

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council" (DHEW Publication No. (N14) 78-23, Revised 1978).

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REPORT

The contract was activated on 1 March 1979 and therefore this report covers the period from the activation date to the final date 28 February 1981; a period of 24 months.

Four publications, listed below, resulted from the work performed.

- 1) Shafer, W. M. and J. J. Iandolo. 1979. The genetics of staphylococcal enterotoxin B in methicillin resistant isolates of staphylococcus aureus. Infection and Immunity 25:902-911.
- 2) Shafer, W. M. and J. J. Iandolo. 1980. Transduction of enterotoxin B synthesis: Establishment of the toxin gene in a recombination deficient mutant. Infection and Immunity 27:280-282.
- 3) Dyer, D. W. and J. J. Iandolo. 1981. Plasmid-chromosomal transition of genes important in staphylococcal enterotoxin B expression. Infection and Immunity 33:450-458.
- 4) Iandolo, John, J. and David W. Dyer. 1982. The staphylococcal enterotoxins - a genetic overview. J. Food Safety 3:249-264.

In addition three contributed papers were presented at scientific meetings; two before the American Society for Microbiology and one before the Missouri Valley Branch of the ASM. The papers given in Los Angeles (ASM) and Dallas (ASM) were titled:

- 1) Shafer, W. M. and J. J. Iandolo. 1979. Demonstration of plasmid and chromosomal genes responsible for staphylococcal enterotoxin B synthesis in methicillin resistant isolates. Bacteriol. Proc. p. 214 (abst.).
- 2) Dyer, D. W. and J. J. Iandolo. 1981. Plasmid involvement in the regulation of staphylococcal enterotoxin B production. Bacteriol. Proc. p. 52.
- 3) Iandolo, J. J. 1981. Staphylococcal enterotoxin B: A genetic overview, Bacteriol. Proc. XXVI.

The staff involved in the project and paid by contract funds were:

- 1) Marilyn I. Rock - Technician
- 2) Margaret (Molly) May - Technician
- 3) William W. Shafer - Graduate student
- 4) David W. Dyer - Graduate student
- 5) Rodney K. Tweten - Graduate student

1. Statement of the problem:

The enterotoxins produced by the Gram positive pathogenic bacterium, Staphylococcus aureus are a group of extracellular proteins (1, 2) that are responsible for the clinical symptomology of staphylococcal food poisoning. Although many cases of staphylococcal food poisoning probably go unreported, epidemiological data reveals that these enterotoxins account for over 25% (1) of all reported food poisoning incidents. While the immediate manifestations of staphylococcal food poisoning are obvious to the clinician, any additional roles that these proteins play during infection remain unknown. However, enterotoxigenic strains have been isolated from patients with chronic osteomyelitis (3), pseudomembranous enterocolitis (4) and scalded skin disease (5, 6).

Research efforts to date have been primarily concerned with enterotoxin purification (7, 8, 1, 9, 10, 11, 12, 13), detection (14, 9, 15, 16, 17, 18) and the regulation of toxin synthesis (19, 20, 21, 22, 23, 24). While these studies have contributed a considerable amount of information regarding enterotoxin biochemistry and synthesis, they have not advanced understanding of the molecular genetics of toxin synthesis. Consequently, there exists a dearth of information regarding the genetics of enterotoxin synthesis. Such information could reveal molecular interrelationships of the various enterotoxins, the transmissibility of the enterotoxigenic phenotype and linkage relationships with genes responsible for antibiotic resistance or in the production of proteins involved in pathogenesis.

The present study was undertaken in order to clarify some aspects of the molecular genetics of enterotoxin synthesis. Although six distinct enterotoxins have been purified, enterotoxin B (SEB) was chosen for genetic analysis because previous work by others (25, 26, 27) suggested that the SEB structural gene was extrachromosomal and was linked to genes responsible for antibiotic resistance.

The results obtained during this study reveal that the SEB gene is capable of existing in two distinct configurations. In a majority (71%) of enterotoxigenic isolates, the gene was found to be restricted to a chromosomal locus whereas in other strains, notably hospital isolates resistant to methicillin, the gene gave evidence of being associated with a 1.15×10^6 dalton plasmid deoxyribonucleic acid (DNA) species. Additional studies demonstrated that the toxin phenotype and by implication, the toxin gene, could be transmitted to previously nontoxigenic strains of S. aureus. The establishment of the enterotoxin gene appeared to be contingent upon the concomitant transfer of genes responsible for methicillin and tetracycline resistance. Although genetic studies demonstrated that the enterotoxin B gene is not closely linked to the antibiotic resistance genes, the data obtained suggest that these genes are capable of a transient association.

2. Background

Early evidence suggesting a plasmid locus for the enterotoxin B gene (entB) was provided by Sugiyama et al. (28). They noticed that when enterotoxigenic isolates which produced a single serotype were plated onto agar containing specific antiserum the SEB⁺ or SEC⁺ colonies produced very

large immune precipitate halos that varied in size. However, SEA⁺ colonies gave comparatively smaller halos which did not vary in diameter. The size heterogeneity of the SEB and SEC immune precipitate halos suggested that these phenotypes were variable and hence unstable while the constant size of the SEA halos indicated that this phenotype was stable. These observations were consistent with other reports which have demonstrated that mutants producing variable amounts of SEB are obtainable without mutagenesis (i.e., occur spontaneously) (29). Conversely, Friedman and Howard (30) were able to obtain mutants which produced high amounts of SEA only after repeated mutagenesis.

Based on the apparent phenotypic stability of SEA synthesis and the instability of SEB and SEC synthesis, Bergdoll et al. (2) proposed that SEA is a chromosomal gene product while the other two serotypes were thought to be plasmid gene products. Subsequent work from my laboratory has confirmed a chromosomal locus for entA (31). However, the genetics of SEB synthesis is more complex (27, 57, 32, 33-Appendix I) and will be discussed in more detail. The genetics of SEC₁ and SEC₂ synthesis remain largely unresolved.

Conceptually, entB could exist in three distinct configurations. The gene could be chromosomal, plasmid or as part of a prophage genome. Each of these three possibilities have been investigated.

A bacteriophage involvement in toxin synthesis in other genera is well documented and is perhaps best typified by the diphtheria toxin model (34). Specifically, the structural toxin gene is present on a bacteriophage genome. Lysogenization of nontoxigenic strains of Corynebacterium diphtheriae confers toxin synthesis and the loss of toxin synthesis is correlated with the loss of immunity to superinfection. Both examples are suggestive evidence of a prophage involvement in toxin synthesis. However, the in vitro synthesis of diphtheria toxin using phage DNA directly demonstrated that the toxin is coded by viral DNA sequences (35). Evidence for a phage involvement in the synthesis of the Escherichia coli heat labile (LT) enterotoxin has also been presented (36).

Against this background, the potential for a phage involvement in SEB synthesis should be addressed. Read and Pritchard (37) examined this question and concluded that although enterotoxigenic staphylococci are lysogenic there does not appear to be a phage involvement in toxin synthesis since lysogenization of nontoxigenic recipients does not confer SEB synthesis. Although fairly detailed their work does not absolutely rule out the possibility of a defective phage involvement since their assay system was restricted to inducible prophage. Nevertheless, the overwhelming data concerning the genetics of SEB synthesis indicates that in at least a majority of strains the determinant is bacterial. However, the recent finding of Takeda and Murphy (36) that the LT enterotoxin gene has migrated from a plasmid to a phage genome in at least two enterotoxigenic strains may provide a renewed stimulus for the further examination of a phage involvement in SEB synthesis.

Initial reports regarding the physical disposition of the enterotoxin B gene (entB) were provided by Dornbusch et al. (25, 38). This group analyzed SEB synthesis in the methicillin resistance (Mec^r) isolate, S. aureus DU-4916. Genetic manipulations involving transduction and elimination of Mec^r suggested linkage of entB with the resistance determinant (mec).

Specifically, methicillin sensitive (Mec^S) recipients rendered Mec^R by transduction virtually always produced SEB. Additionally, all mec^S derivatives, obtained by cultivation in the presence of the curing agent acriflavine, lost the SEB phenotype. Based on these results, Dornbusch et al. (25, 38) proposed the entB and mec are linked on a plasmid. Unfortunately, they did not provide any biophysical data to substantiate the presence of such a plasmid species.

The finding of a potential plasmid involvement in Mec^R provided a stimulus for other studies which were devoted to the elucidation of the genetics of resistance. Cohen and Sweeney (39) reported that unlike the findings of Dornbusch et al. (25, 38) the Mec^R phenotype gave evidence of being coded for by a chromosomal gene since the phenotype could not be eliminated. Stiffler et al. (40) and Kayser et al. (41) examined the extrachromosomal DNA profile of Mec^R isolates and resistant transductants but were unable to isolate a plasmid DNA species equatable with resistance. These groups suggested that unless mec is harbored by a unique plasmid, which is not detectable by standard plasmid identification techniques, the gene should be considered chromosomal.

The most convincing data implicating a chromosomal mec gene was provided by Sjöström et al. (42) and Kuhl et al. (43). Sjöström et al. demonstrated that chromosomal DNA but not plasmid DNA preparations were effective in transforming Mec^S recipients to the resistant phenotype. Additionally, mec transformation occurred in both recA⁺ and recA⁻ recipients. Regardless of the recipient, the gene was apparently established in the chromosome since no plasmid DNA equatable with Mec^R could be isolated from the Rec^- Mec^R transformants. Since recombination did not require a functional recA gene product, Sjöström et al. (42) stated that although mec is chromosomal it should be considered a pseudoplasmid, as defined by Wyman et al. (44).

Kuhl et al. (43) confirmed the chromosomal disposition of mec and established that the gene, regardless of the isolate, always maps within linkage group II of the staphylococcal chromosome. By a series of three factor crosses mec was shown to map next to the determinants responsible for novobycin resistance and purine biosynthesis. Because of this linkage arrangement, Kuhl et al. questioned whether the determinant was capable of transposition, as proposed by Sjöström et al. (42). Unfortunately, they did not employ recA⁻ recipients and as such, the requirement for a functional recA gene product was not ascertained. Since the staphylococcal chromosome remains poorly defined their data does not eliminate the possibility that mec may also exist in other areas of the genome.

None of the previously described studies involved in detailing the genetic locus for Mec^R employed SEB as a secondary marker and the proposed linkage (25, 38) of these two determinants was not resolved. Since the original reports of Dornbusch et al. (25, 38), other reports (27, 31, 33-Appendix I, 27) have appeared dealing with the genetics of SEB synthesis. These studies have been concerned with ascertaining the disposition of the entB gene (i.e., plasmid or chromosomal) and the linkage arrangement of entB and mec. The cumulative data suggests that the genetics of SEB synthesis is quite complicated in that the toxin determinant is capable of exhibiting genetic but not physical linkage with genes responsible for Mec^R and tetracycline resistance (Tc^R) (27, 33-Appendix I) and can exist in either the plasmid and chromosomal state (32, 33-Appendix I, 38).

The work of Shalita et al. (27) represented the first indepth biochemical analysis of the genetics of SEB synthesis. Like Dornbusch et al. (25, 38), this group examined entB in strain DU-4916. Extrachromosomal DNA analysis by neutral sucrose gradient centrifugation of labeled cleared lysates demonstrated that strain DU-4916 harbors three distinct plasmids; 37S, 21S, and 14S. Contour mapping of the 14S plasmid revealed that the molecule had an apparent molecular weight of 0.75×10^6 daltons. Previous investigators (45, 42, 40) did not report the presence of this plasmid in strain DU-4916 but careful inspection of their data indicates that gradient centrifugation profiles contain this small plasmid, which may have appeared as a "shoulder" of the 21S plasmid.

Marker analysis performed by transduction and elimination demonstrated that the 37S plasmid harbored determinants responsible for penicillin and cadmium resistance while the 21S molecule was responsible for tetracycline resistance (Tc^r). Transduction studies demonstrated that neither entB or mec is linked to either of these plasmids. Ethidium bromide curing experiments, however, demonstrated that the loss of either Mec^r or Tc^r , or both, resulted in the concomitant loss of SEB synthesis. Plasmid DNA analysis of the SEB⁻ derivatives showed that all of the clones lost the 0.75×10^6 dalton plasmid. Extrachromosomal DNA analysis of various transductants demonstrated SEB⁺ clones contained the 0.75×10^6 dalton plasmid species while the SEB⁻ clones contained only the various antibiotic resistance plasmids. Based on the plasmid DNA analysis of the entB genetic derivatives, Shalita et al. proposed that the 14S plasmid contains a genetic determinant critically involved in SEB synthesis.

Since the SEB⁺ phenotype cannot be directly selected in genetic manipulations it is possible that the presumptive entB plasmid only harbors a determinant involved in the regulation of toxin synthesis and not necessarily the structural entB gene. To be sure, the inability to directly select for enterotoxigenic clones has proved to be a major hinderance in studies dealing with entB genetics.

Shafer and Iandolo (32) have confirmed the existence of a 14S plasmid in strain DU-4916 and demonstrated that this extrachromosomal species is absent in a nontoxigenic methicillin sensitive derivative (DU-4916S). Velocity centrifugation of labeled cleared lysates in neutral sucrose gradients indicated that the putative entB plasmid has an apparent molecular weight of 1.15×10^6 daltons. A similar molecular weight was obtained when the bulk plasmid DNA obtained from DU-4916 were electrophoresed with staphylococcal plasmids of known molecular weight in agarose gels (33-Appendix I).

Shafer and Iandolo (32) reported that although entB appears to be associated with the 1.15×10^6 dalton plasmid in strain DU-4916 the gene also exists as a chromosomal entity in other isolates. Analysis of the plasmid DNA profile of five methicillin sensitive SEB⁺ isolates failed to reveal the presence of the putative entB plasmid. Moreover, genetic examination of the plasmid DNA harbored by these strains failed to reveal association of entB with any of the resident plasmid DNA species. The combined biophysical and genetic results indicated that these isolates contained a chromosomal entB gene.

The reports of Dornbusch et al. (25, 38), Shalita et al. (27) and Shafer and Iandolo (32, 33-Appendix I) have established that entB can exist in either the plasmid or chromosomal state. However, the relationship between mec and entB is less clear. Cotransduction and coelimination results strongly argue that both genes are closely linked. However, the rigorous examination of mec in S. aureus DU-4916 unequivocally demonstrated that the gene is chromosomal (43, 42), while, entB appeared to be harbored by the 1.15×10^6 dalton plasmid. Based on the apparent physical disposition of these two genes, the observed linkage appears to be paradoxical.

3. Approach to the problem

In the initial portion of this work, we addressed the question of linkage of methicillin resistance and enterotoxin B production using standard genetic techniques consistent with the state of the art in the staphylococcal genetic system. Transductional and transformational crosses were carried out to sort out the very complex relationships involved. All these were verified by biophysical analysis of the recipient derivatives.

The first question asked was whether the putative enterotoxin B plasmid was common to methicillin resistant strains of S. aureus. To answer this question a survey of the plasmid make-up of 16 mec^r SEB⁺ strains was performed. Detailed genetic analysis of four of these strains was carried out by transduction and transformation. Because the data suggested that entB may be a transposon further studies involving a recombination deficient mutant were carried out to test this possibility.

We also wished to know whether one of the sequelae of natural drug transmission among these strains was the cotransfer of toxin determinants. To answer this question we cocultivated clearly distinguishable genetically marked strains, one of which (the donor) produced SEB and selected for toxin producing variants of the recipient.

Lastly, we spent the past year examining the function of pSN2, the putative enterotoxin plasmid. To do this we employed transformation into heterogenetic hosts, minicell and in vitro translation assays and standard genetic techniques to construct strains useful in the analysis. All constructed strains were verified by agarose gel electrophoresis and the minicell and in vitro assays were verified by SDS-PAGE analysis.

4. Results and discussion

The details of the genetic analysis of entB is contained in Appendices 1, 2, and 3. The reader is directed to these for a more in-depth discussion. However, a summary of these findings is presented here.

Biophysical and genetic analysis (appendix 1) of staphylococcal enterotoxin B (SEB) synthesis in 16 methicillin-resistant (Mec^r) S. aureus isolates demonstrated that the toxin gene (entB) can occupy either a plasmid or a chromosomal locus. Biophysical analysis of the plasmid DNA content of these

strains by agarose gel electrophoresis revealed the presence of a 1.15 megadalton (MDAL) plasmid in six isolates (37.5%) that appears to contain the entB gene. Genetic manipulation of SEB synthesis by transduction and elimination demonstrated that this plasmid is critical for enterotoxigenesis. Nevertheless, the majority of the Mec^r SEB^+ isolates (63.5%) analyzed in this investigation were found to lack the 1.15 MDAL plasmid. In at least two of these strains (COL and 57-dk) transduction and elimination showed that entB was chromosomal.

Genetic studies involving strains harboring either a plasmid or a chromosomal entB gene (appendix 1) demonstrated that toxin synthesis is coeliminated with mec. However, analysis of the entB and mec loci by transformation or transduction showed that the genes are not closely linked. On the other hand, transduction of entB, regardless of the donor, was observed when mec and Tc^r (tetracycline) plasmid were jointly cotransduced. This finding suggests that during transduction a transient association among entB, mec and the Tc^r plasmid may exist. Because of the genetic duality (plasmid-chromosomal) demonstrated by entB and since the methicillin gene has already been reported (42) to be part of the transposition-like element due to its ability to be established in recombination deficient hosts (recA1⁻), we undertook experiments to determine if entB exhibited recA1 independent recombination (Appendix 2). The results of these experiments demonstrated that only entB from strains containing entB could be established in recA1⁻ hosts. Indeed the plasmid profile of such recipients clearly showed the presence of entB. Chromosomal entB strains could not be successfully employed as donors. Therefore, by this criterion it seems that entB does not exhibit properties of a transposon. However, because of the inability to directly select for entB this experiment may not be entirely adequate to analyze the question of entB transposability. On the other hand, the high cotransfer of entB, mec and tc (85% of mec^r Tc^r clones are SEB^+) argue strongly for the validity of the approach.

In as much as the entB gene could not be shown to undergo recA1 independent recombination we attempted to map the entB gene on the S. aureus chromosome. We had shown (Appendix 1) that entB did not map near mec in linkage group II but had empirically noted that most SEB^+ transformants were also transformed for pigment production (pig⁺). We therefore examined the pig region of linkage group III. DNA from strain COL was used to transform ISP484 (33). Recombinants were selected that were prototrophic for isoleucine, leucine and valine (ilv) and scored for pigmentation. Ilv was transformed at a frequency of 3.8×10^{-9} (38 total clones). Cotransformation of pig was 8% (3 clones) and all of these were SEB^+ . The data thus far are encouraging and hint that entB may map between ilv and pig. However, expression of toxin is strongly subdued (that is; 25X concentration of the medium must be made before SEB can be detected) and transformant clones produce only about 1 μg of SEB/ml. We are continuing mapping studies and studies designed to clarify the genetic associations alluded to in appendix 1.

In an associated study we wished to know whether one of the sequelae of natural drug transmission among these strains was the cotransfer of toxin determinants. To answer this question we cocultivated clearly distinguishable

genetically marked strains, one of which (the donor produced enterotoxin B.) The relevant phenotype of the donor was $mec^r tet^r nov^s SEB^+$ ($\phi 11$), the phenotype of the recipient was $mec^s tet^s nov^r SEB^-$. Recombinants were scored for the $mec^r tet^r nov^r SEB^+$ phenotype. Although transduction frequencies as high as 2×10^4 recombinants per plaque forming unit were observed for single markers, the frequency of the triple transductants was considerably lower but nonetheless did occur. Since lysogenized staphylococci exhibit relatively high amounts of prophage leakiness, this result is not unexpected. However, the high frequencies observed suggest that a higher proportion of leaky phage are transducing particles than are phage from conventional lysates. Furthermore, this result provides evidence of genetic promiscuity among naturally associated strains and may provide the mechanism for the contribution to or exacerbation of other staphylococcal disease by enterotoxin.

We have begun studies to investigate the genetic disposition of enterotoxin C in S. aureus. Though the generous gift of SEC₂ from Dr. R. Bennett (FDA Washington, D.C.) we have prepared antibody for analysis in a manner similar to that reported by us for SEB (32). SEC₁ and SEC₂ can be analyzed with this serum by Laurell gel electrophoresis with about the same sensitivity as SEB.

Three strains were initially chosen for analysis (strains 735, 740 and 834. These strains were also obtained from Dr. R. Bennett, FDA, Washington). Strains 735 and 834 produced both SEC₁ and SEC₂ while strain 740 produced SEC₂. All three produced enterotoxin C at about 50 μ g/ml. Biophysical analysis of the plasmid DNA of these three strains by CsCl ethidium bromide centrifugation followed by agarose gel electrophoresis demonstrated that all three possess a single plasmid species of 17.5×10^6 daltons. Analysis of this plasmid in all three strains has shown it to confer cadmium and penicillin resistance. In relationship to patterns already established we presume this is tentative evidence of a chromosomal locus for SEC₂. Work is continuing on a more critical analysis of SEC production.

Work carried out this past year was intended to clarify the role of pSN2 in SEB synthesis. (The details of these data are presented in Appendix 3.) This was necessary since this laboratory has shown (Appendix 1) that the majority of SEB⁺ isolates examined produce SEB without harboring this 1.15 Mdal plasmid, yet pSN2 appears essential for enterotoxigenesis in those strains carrying the replicon. We approached the resolution of this apparent controversy by directly evaluating the phenotypic expression and translation products of pSN2 in the heterogenetic background provided by B. subtilis. A number of staphylococcal plasmids, such as p194, pE194, and pUB110, have been shown to be expressed with the appropriate phenotype in B. subtilis. However, when we placed pSN2 into B. subtilis (Appendix 3, Fig. 1A) SEB production was not observed in the transformant clones. This might be expected if pSN2 were not fully expressed in B. subtilis or, alternatively, if the plasmid did not contain the enterotoxin structural gene. Since transfer of both pC194 and pSN1 into these recipients produced the appropriate phenotype (one

transformant, KSI503, Appendix 3, Table 1, was $\text{Cm}^r \text{Tc}^r$), it seems likely that there is no barrier to the expression of pSN2 in these cells. Since pSN2 presented us with a cryptic phenotype (that is, since the *B. subtilis* transformants were SEB^-) it was necessary to determine if proteins were produced by pSN2 in this heterogenetic host. We therefore transformed pSN2 into a minicell producing strain of *B. subtilis* (Appendix 3, Fib. 1B). On the basis of the *B. subtilis* minicell (Appendix 3, Fig. 2) and the *E. coli* in vitro assays (Appendix 3, Fig. 2) we concluded that the plasmid was expressed in these systems, but that SEB was not produced. Unpublished data from Novick' group (R. P. Novick, Public Health Institute of the City of New York) citing experiments similar to ours suggests that pSN2 specified a 20,000 dalton protein and an 11,000 dalton protein. We assume that the 20,000 dalton protein seen in their experiments is that identified by us as having a molecular weight of 18,000 daltons. In some in vitro translation experiments we have also seen a second polypeptide of about 11,000 daltons that appears to be pSN2-specific. We have been unable to consistently reproduce these results and are consequently unsure about the origin of this 11,000 dalton protein. Additionally, unpublished nucleotide sequence data of Novick indicate that codons corresponding to the amino acid sequence of SEB do not reside on the majority (approx. 2/3) of pSN2. However, if the structural SEB gene is present in pSN2, it must require factors peculiar to the staphylococcal protein synthetic machinery for expression. If the plasmid requires a function provided by any staphylococcal cell for SEB synthesis, then *S. aureus* strain KSI400 (an RN450 derivative containing pSN2, Appendix 3, Table 1) should have been enterotoxigenic. However, KSI400 remained SEB^- . Therefore, it seems unlikely that the structural gene for SEB resides on pSN2 but suggests that expression of the SEB^+ phenotype, if dependent upon pSN2, requires a second unlinked gene (the structural SEB gene) not present in *S. aureus* RN450.

An obvious alternative to this interpretation is that this cryptic plasmid is not involved in SEB synthesis. In this instance, the association between pSN2 and the SEB^+ phenotype shown by this laboratory (Appendix 1, 2) and by others (25, 27) cannot be easily explained. However, the possibility that pSN2 is not involved in SEB synthesis led us to reexamine whether the plasmid was actually necessary for enterotoxigenesis in *S. aureus* DU-4916. In attempting to cure pSN2 from DU-4916 with ethidium bromide, we found (Appendix 3, Table 2) a spontaneous segregation (ca 50%) of this strain to the SEB^- phenotype. Notwithstanding the ethidium bromide treatment, the SEB^- phenotypic segregation occurred in the absence of any demonstrable effect on the intracellular residence of pSN2. These SEB^- DU-4916 segregants display a phenotype similar to KSI400. Both strains contain pSN2, yet are SEB^- . The high rate of spontaneous segregation of SEB^- we have seen is startlingly different than the curing observed by Dornbusch et al. (25) of Mec^r and SEB^+ phenotypes. These authors observed a coelimination of the two markers (12.5% of acriflavine-treated cells). Neither the Mec^r nor SEB^+ phenotypes were spontaneously lost. The spontaneous SEB^- derivatives we have isolated continue to be resistant to methicillin. However, our data may explain the coelimination of the Mec^r and SEB^+ phenotypes, without suggesting a direct genetic link between the two. If one assumes that segregation of SEB proceeds at a rate of about 97% after acriflavine treatment

(as we have shown for ethidium bromide-treated cells), then Mec^S cells arising in the culture have a high probability of being SEB^- .

One might use this same argument regarding the curing of pSN2 from DU-4916 wherein the resulting derivative, KSI393 (Appendix 3, Tables 1, 2) became SEB^- . Since we have shown that DU-4916 segregates the SEB^- phenotype at a high rate, one might predict in any event that KSI393 (Appendix 3, Table 1) would become SEB^- . This strain, although lacking pSN2, could be SEB^- due to the fact that it was a spontaneous SEB^- segregant. While this occurred with many clones, many others were not spontaneous segregants because when minicells containing pSN2 were fused with KSI393, the recipients often became SEB^+ (Appendix 3, Fig. 3A, B). It is difficult to explain how this could occur without concluding that pSN2 is required for enterotoxigenesis in strain DU-4916. The fact that many Cm^r SEB^- colonies were isolated in this experiment can be explained as the result of two processes. Firstly, if all minicells did not contain both pC194 and pSN2 one would not expect a complete correspondence between the Cm^r and SEB^+ phenotypes. Secondly and more importantly, SEB^- cells could arise if instability in the SEB^+ phenotype in DU-4916 is due to a second element required for enterotoxigenesis. If this segregation occurred in the absence of pSN2, that cell would remain SEB^- even after introduction of the plasmid into the cell.

Our data also indicate that pSN2 is required for SEB synthesis in chromosomal SEB producers as well as in plasmid-bearing strains. This was initially inferred from the fact that KSI393 Cm^r SEB^+ recipients in minicell fusion experiments contained neither pC194 nor pSN2 as autonomous replicons (Appendix 3, Fig. 3A, B). Both elements apparently became chromosomally incorporated. At present we can offer no explanation for this result. Originally, pSN2 could certainly replicate autonomously in strain DU-4916. One might suggest that the lack of autonomy displayed by the plasmids was an artifact of the protoplast fusion technique. This is quite possible, since the same experimental conditions were used to cure pSN2 from DU-4916 and to reintroduce the plasmid. However, this explanation is difficult to reconcile with the fact that a similarly constructed strain, KSI400, contains autonomous pC194 and pSN2 replicons (Appendix 3, Fig. 3A, B).

In accordance with the suggestion that pSN2 could support SEB synthesis from a chromosomal site in KSI393, we found that an SEB^- DU-4916 derivative containing pSN2 (KSI392) was able to complement a chromosomal SEB^- derivative in order to produce SEB (Appendix 3, Table 3A, B). In these experiments we detected no autonomous plasmids in the recipient SEB^+ cells which would correspond to pSN2 (Appendix 3, Fig. 3A, B). Since this plasmid did not appear in the recipient, we infer that a pSN2 element must be capable of integration into the recipient chromosome in order to support SEB synthesis. It would be difficult to support this contention without hybridization data, except that we have shown that pSN2 is capable of integrating in the DU-4916 chromosome in order to support SEB synthesis. We are at present conducting hybridization experiments using the Southern technique in order to confirm the chromosomal site of pSN2 in strain S6 and to study the manner in which integration occurs. The fact that pSN2 becomes chromosomally situated during transformation (in addition to the protoplast fusion experiments), where

cell membrane alterations should be minimal, underscores the suggestion that integration is unrelated to the protoplast fusion technique used in previous experiments.

What is the role of pSN2 in SEB synthesis? We propose that pSN2 regulates SEB synthesis by affecting the expression of the SEB structural gene present on the chromosome of certain S. aureus strains. This function (or functions) is expressed whether the plasmid remains an autonomous replicon or becomes chromosomal. Any discussion of the nature of the regulation of SEB synthesis by pSN2 would be highly speculative at the present time.

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Genetics of Staphylococcal Enterotoxin B in Methicillin-Resistant Isolates of *Staphylococcus aureus*[†]

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Biophysical and genetic analysis of staphylococcal enterotoxin B (SEB) synthesis in 16 methicillin-resistant (*Mec*^r) *Staphylococcus aureus* isolates demonstrated that the toxin gene (*entB*) can occupy either a plasmid or chromosomal locus. Biophysical analysis of the plasmid deoxyribonucleic acid content of these strains by agarose gel electrophoresis revealed the presence of a 1.15-megadalton plasmid in six isolates that appears to contain the *entB* gene. Genetic manipulation of SEB synthesis by transduction and elimination procedures demonstrated that this plasmid is critical for enterotoxigenesis. Nevertheless, the majority of the *Mec*^r SEB⁺ isolates (62.5%) analyzed in this investigation were found to lack the 1.15-megadalton plasmid. In at least two of these strains (COL and 57-dk), transduction and elimination procedures showed that *entB* was chromosomal. Genetic studies involving strains harboring either a plasmid or chromosomal *entB* gene demonstrated that toxin synthesis was coeliminated with *mec*. However, analysis of the *entB* and *mec* loci by transformation or transduction showed that the genes are not closely linked. On the other hand, transduction of *entB*, regardless of the donor, was observed when both *mec* and the Tc^r plasmid were jointly cotransduced. This finding suggests that, during transduction, a transient association between *entB*, *mec*, and the Tc^r plasmid may exist.

Studies dealing with the genetics of staphylococcal enterotoxin B (SEB) biosynthesis indicate that the toxin gene (*entB*) may be either plasmid or chromosomal (3, 4, 17, 19). Dornbusch et al. (3, 4) studied the genetics of SEB synthesis and reported that, in the methicillin-resistant (*Mec*^r) isolate *Staphylococcus aureus* DU-4916, the *entB* and *mec* determinants were cotransduced and coeliminated at frequencies suggestive of linkage on a small plasmid. As a result, the *mec* gene became the subject of intense investigation, and the cumulative data have unambiguously shown that this determinant is chromosomal (6, 8, 19, 20), maps within *pyr-his-nov-pur-mec* linkage group (8), and exhibits properties of a pseudoplasmid (19, 20, 23).

The *entB* gene was not included in those investigations but, using the *Mec*^r strain of Dornbusch et al., Shalita and co-workers (19) reinvestigated the genetics of SEB synthesis. They provided biochemical and genetic data which strongly implicated the involvement of a small plasmid (*pentB*) in SEB production. Whether this plasmid contains the *entB* structural gene or some determinant critical for enterotoxi-

genesis was not determined, although the latter seems an unlikely alternative because of the complete correlation observed.

We (17) have been able to confirm the data of Shalita et al. (19) in the *Mec*^r strain DU-4916, but in five methicillin-sensitive strains we were unable to detect the presumptive SEB miniplasmid. Genetic analysis of the plasmid deoxyribonucleic acid (DNA) in these *Mec*^r strains failed to reveal a plasmid *entB* locus, and SEB synthesis was shown to be directed by chromosomal determinants.

This study of the *entB* gene was undertaken to further clarify the genetic status of SEB synthesis. Specifically, we have been concerned with the disposition of *entB* in *mec* isolates because of the reported genetic linkage of these two determinants (3, 4). Additionally, we have also been concerned with the frequency with which *pentB* is present among enterotoxigenic *Mec*^r isolates. The results presented in this communication demonstrate that the toxin determinant in *Mec*^r isolates can be either plasmid or chromosomally borne but, in either case, is not physically linked to the *mec* gene as previously suggested (3, 4). However, transduction results suggest a transient association of *entB* with the *mec* gene and a tetracycline resistance plasmid.

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MATERIALS AND METHODS

Organisms. The *S. aureus* strains employed in this investigation are listed in Tables 1 and 2.

Media. For enterotoxin analysis, the various strains were grown in 3% NP broth (5). Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) was used for routine culture media, phage propagation, and as the growth media in ethidium bromide curing experiments (17, 18). When agar plates were required, 1.5% agar (Difco Laboratories, Detroit, Mich.) was added to either TSB or NP broth.

Marker analysis. Cadmium nitrate [$\text{Cd}(\text{NO}_3)_2$] sensitivity was determined as previously described (17, 21). Antibiotic sensitivity was determined as described by Dornbusch et al. (3, 4), with the *Mec*^r phenotype scored at 30°C or at 37°C in the presence of 5% NaCl.

Enterotoxin B production and assay. Conditions for maximal enterotoxin synthesis have been described (5). Analysis of SEB production was performed by Laurell immunoelectrophoresis (5) or gel double diffusion (15). When necessary, supernatants were concentrated by pervaporation. Monospecific antiserum was prepared in New Zealand white albino rabbits using highly purified SEB (kindly provided by R. Bennett, U. S. Food and Drug Administration, Washington, D.C.). SEB production by individual colonies was analyzed by replica plating onto NP agar containing a 1:40 dilution of anti-SEB serum or by Elek plate (1) analysis. Normal rabbit serum controls were also run to distinguish nonspecific immunoprecipitation due to protein A interaction.

Genetic manipulations. (i) **Transduction.** All transduction experiments were performed with phage 29 of the International Typing Series that were plaque purified on the donor strain. Transducing lysates were prepared on the appropriate donor strains and sterilized as previously described (16, 17). The phage dilution buffer was used according to Novick (13). Transduction was carried out (3, 4, 17) with multiplicities of infection ranging from 0.1 to 1.0. The recipient strain for all transduction experiments was *S. aureus* 8325-4(ϕ 11) or derivatives of this strain. After 2 h of preincubation at 37°C or 4 h at 30°C, transductants were selected by the antibiotic soft agar overlay procedure of Sjöström et al. (20). The concentrations of the selective agents were as follows: cadmium nitrate, 25 $\mu\text{g}/\text{ml}$; tetracycline (Tc), 5 $\mu\text{g}/\text{ml}$; methicillin (*Mec*), 5 $\mu\text{g}/\text{ml}$; novobiocin (*Nov*), 10 $\mu\text{g}/\text{ml}$; and penicillin G (*Pe*), 10 $\mu\text{g}/\text{ml}$. Further incubation was carried out at 37 or 30°C for 48 to 72 h before scoring for transductants. The transduced phenotype was confirmed by replica plating onto Trypticase soy agar (TSA) plates containing the appropriate selective agent. Uninfected and phage sterility controls were always included.

(ii) **Transformation.** Transforming chromosomal DNA in sterile 0.1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) was prepared as described by Sjöström et al. (20) and used for *mec* transformation (8, 20). DNA concentrations were determined by ultraviolet absorbance at 260 nm. Controls consisted of individually plating the transforming DNA and untreated cells onto selective agar plates. Incubation of transforming DNA with deoxyribonucle-

ase (20) resulted in the complete loss of transforming activity.

(iii) **Marker elimination.** Ethidium bromide curing experiments were performed as previously described (17, 18). Spontaneous elimination of a particular marker was determined by plating approximately 100 colony-forming units per plate (after sonication to disrupt clumps) onto TSA and then replica plating onto selective or antiserum agar.

The spontaneous elimination of either the *Mec*^r or *SEB*^r phenotype was not observed in strains DU-4916, 592, C91, or 57-dk when even 500 colonies grown on TSA plates were replica plated to methicillin and antiserum agar plates. Therefore, the isolation of the negative variants was due to the elimination procedures used.

Analysis of plasmid DNA. The *S. aureus* strains were propagated in 50 ml of TSB at 37°C for 24 h, and cleared lysates were produced (14, 17). The volume of the cleared lysates was doubled with chloroform-isoamyl alcohol (24:1), and multiple extractions were carried out by shaking at 37°C for 5 min followed by 30 min at 4°C. The aqueous phase was collected after centrifugation at $5,000 \times g$ for 30 min at 4°C. A clear aqueous phase was generally present after two to three extractions. Generally, ribonuclease treatment (7, 12) of the lysates was not necessary. After the final extraction, two volumes of cold 95% ethanol were added and the cleared lysate was stored at -20°C for at least 4 h to precipitate plasmid DNA. The DNA precipitates were collected by centrifugation at $12,000 \times g$ for 20 min at -10°C. The ethanol was carefully drained, and the pelleted material was gently redissolved in 0.2 ml of 0.03 M tris(hydroxymethyl)aminomethane-0.0025 M ethylenediaminetetraacetic acid-0.05 M NaCl (pH 8.0).

Plasmid DNA species from cleared lysates were analyzed by horizontal gel electrophoresis. For electrophoresis, 1% agarose gels (Seakem HGT Agarose, Marine Colloids) were run at 60 V and 28 mA. The electrophoretic run lasted 4.5 h or until the tracking dye (12) reached the end of the gel. The electrophoresis buffer consisted of 0.089 M tris(hydroxymethyl)aminomethane, 0.089 M boric acid, and 0.0025 M ethylenediaminetetraacetic acid. Generally, 10 to 50 μl of the plasmid DNA solutions was analyzed. After electrophoresis, the gels were stained with a 0.5 $\mu\text{g}/\text{ml}$ solution of ethidium bromide for 30 min and photographed over a short-wave ultraviolet transilluminator using Polaroid type 55 P/N film. The electrophoretic position of each plasmid species in the agarose gels was checked with CsCl-ethidium bromide density gradient-purified plasmid DNA (12, 17). To purify individual plasmid species, preparative gel electrophoresis was carried out by placing an entire cleared lysate in a trough in the gel and subjecting it to electrophoresis under the same conditions as the analytical technique. The desired plasmid band was cut from the gel and electrophoretically eluted into dialysis sacs. The purified plasmid preparation was then dialyzed against electrophoresis running buffer for 24 h, precipitated with ethanol, redissolved in 0.03 M tris(hydroxymethyl)aminomethane-0.0025 M eth-

TABLE 1. Designation, phenotype, and origin of *S. aureus* test strains

Strain	Relevant phenotype ^a	Origin
DU-4916	Pc ^r , Tc ^r , Mec ^r , SEB ⁺ (17.5)	S. Cohen
DU-4916S	Pc ^r , Tc ^r , Mec ^r , SEB	S. Cohen
COL	Tc ^r , Mec ^r , SEB ⁺ (3.0)	B. Wilkinson
57-dk	Pc ^r , Tc ^r , Mec ^r , SEB ⁺ (17.5)	B. Wilkinson
592	Pc ^r , Tc ^r , Mec ^r , SEB ⁺ (17.5)	B. Wilkinson
8325-4 (φ11)	Mec ^r , SEB	P. A. Pattee
ISP2	Nov ^r , Mec ^r , SEB	P. A. Pattee
RN 492	Pc ^r , SEB	R. Novick
8325-4 (pC194)	Cm ^r , SEB	E. Lederberg, PRC ^b
8325-4 (pT169)	Tc ^r , SEB	E. Lederberg, PRC ^b

^a Marker abbreviations are as follows: Pc^r (penicillin resistance), Tc^r (tetracycline resistance), Mec^r (methicillin resistance), SEB⁺ (production of enterotoxin B), Nov^r (novobiocin resistance), Cm^r (chloramphenicol resistance).

^b Expressed as micrograms per milliliter of SEB produced by 36 h shake cultures as determined by Laurell immunoelectrophoresis (see text).

^c PRC, Plasmid Reference Center, Stanford, Calif.

ylenediaminetetraacetic acid-0.05 M NaCl (pH 8.0), and stored at -20°C until used.

RESULTS

The *entB* gene is a chromosomal determinant in all of the Mec^r SEB⁺ isolates of *S. aureus* we have so far examined (17). On the other hand, the Mec^r SEB⁺ isolate, DU-4916, has been shown by Shalita et al. (19) and verified by us (17) to possess a small plasmid that apparently contains the *entB* gene. Since enterotoxigenicity is characteristic of most Mec^r strains (10), we wished to determine if the plasmid *entB* genotype was characteristic of Mec^r SEB⁺ strains. Cleared lysates of 16 such strains were produced and subjected to agarose gel electrophoresis. In Fig. 1, an agarose gel profile of the plasmid DNA from four test Mec^r SEB⁺ isolates (592, COL, DU-4916, and 57-dk) is presented. The banding patterns of the pentB plasmid (1.15 megadaltons [Mdal]), isolated and purified from strain DU-4916 by preparative gel electrophoresis, and of chromosomal DNA from the plasmid-negative strain 8325-4(φ11) are also presented. Strains DU-4916 and 592 were found to contain a plasmid species migrating at 1.15 Mdal (Fig. 1). These two strains were also found to contain a 17.5- and a 3.0-Mdal plasmid responsible for Pc^r and Tc^r, respectively (Fig. 1). Molecular weights of the plasmid species were determined by the linear migration of reference staphylococcal plasmids (Fig. 2). Strain COL contains only a single 3.0-Mdal plasmid which was shown to encode for Tc^r, whereas strain 57-dk harbors a 3.0-Mdal Tc^r plasmid and a 17.5-Mdal Pc^r plasmid. To ascertain the frequency at which the pentB plasmid is present among such isolates,

we extended this analysis to examine the plasmid DNA profile of 12 additional strains. The plasmid DNA content of all 16 Mec^r SEB⁺ isolates is presented in Table 2. Of 16 isolates examined, only 37.5% (6/16) contained the pentB plasmid. Each of these six also possessed a 3.0-Mdal Tc^r plasmid and a 17.5- to 22.4-Mdal Pc^r plasmid. The remaining strains possessed a varying array of similar plasmid species.

Since agarose gel electrophoresis of cleared lysate DNA resolves not only covalently closed circular DNA, but also the open circular and linear plasmid forms as well as contaminating chromosomal DNA (12), it was necessary to purify Lnk covalently closed circular DNA to verify the band positions of the individual plasmids. This was accomplished by cesium chloride-ethidium bromide equilibrium density gradient centrifugation of [³H]thymidine-labeled cleared lysates (14, 17). The covalently closed circular DNA peak was collected and electrophoresed in 1% agarose gels. The results (not presented) were identical to those already discussed.

The limited distribution of pentB among these Mec^r cultures led us to question the genetic configuration of SEB in toxin-positive strains

TABLE 2. Plasmid DNA profile of Mec^r SEB⁺ strains^a

Strain	Mol wt of plasmids ^b	SEB synthesis ^c
DU-4916	17.5 (Pc ^r), 3 (Tc ^r), 1.15 (SEB ⁺)	50
592	17.5 (Pc ^r), 3 (Tc ^r), 1.15 (SEB ⁺)	25
Kasanjian	22.4, 3.1	30
57-dk	17.5 (Pc ^r), 3.0 (Tc ^r)	23
5814R ^d	22.4 (Pc ^r), 1.8 (Cm ^r)	1.5
Meuse	17.5, 3.1	22
Japan	20, 3	23
Dumas	20, 3, 1.15	21.6
5106R	20, 3	16.7
7074R	20, 3, 1.15	11.9
5619	20, 3, 1.15	14.3
69129	20, 3, 1.15	15.5
COL	3 (Tc ^r)	12.5
456-33	20	18
5205-R	20	3.4
639-45I	20	10.9

^a All strains except for DU-4916 were supplied by B. Wilkinson.

^b Expressed in megadaltons as determined by agarose gel electrophoresis.

^c Expressed as micrograms per milliliter as determined by Laurell immunoelectrophoresis.

^d A Mec^r SEB⁺ derivative (5814S), obtained from B. Wilkinson, was found to contain an identical plasmid profile.

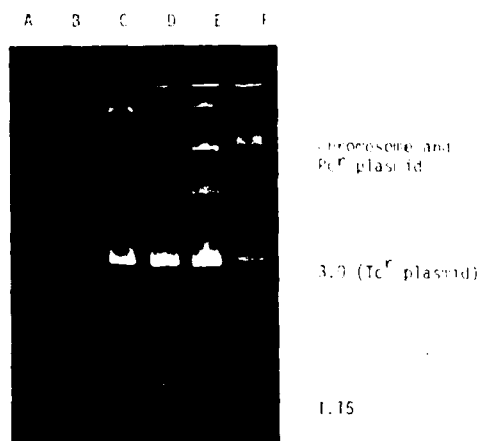


FIG. 1. Agarose gel electrophoresis of ethanol-precipitated cleared lysate DNA from SEB⁺ isolates, strain 8325-4(ϕ 11), and purified SEB plasmid. (A) Electrophoretically purified SEB plasmid; (B) chromosomal DNA from *S. aureus* 8325-4(ϕ 11); (C) *S. aureus* DU-4916; (D) *S. aureus* 592; (E) *S. aureus* 57-dk; and (F) *S. aureus* COL. Bands migrating behind the chromosomal and Pc^r plasmid DNA represent the open circular form of the Pc^r plasmid, whereas bands migrating between the Pc^r and Tc^r plasmids represent the open circular form of the Tc^r plasmid. The position of the covalently closed circular form of each plasmid was confirmed by electrophoresis of cesium chloride-ethidium bromide-purified covalently closed circular DNA.

lacking pentB. Therefore, we analyzed strains 592 and COL. Strain 592 was chosen as a plasmid control because it contained pentB, whereas COL was selected because it did not contain this small plasmid but only a single 3.0-Mdal Tc^r plasmid. Further, analysis of 592 would provide genetic evidence of pentB in a second strain in addition to DU-4916 (17, 19).

Previous genetic manipulation of SEB⁺ strains has demonstrated that the *entB* gene is not physically linked to plasmids encoding for resistance to cadmium, penicillin, or tetracycline (3, 4, 9, 17, 19). However, cotransduction of *entB* with the Tc^r plasmid and the *mec* gene from strain DU-4916 has been observed (3, 4, 19). Therefore, as a strategy in genetic evaluation of *entB* linkages, we employed transduction of the plasmid Tc^r marker and analyzed for cotransduction of *mec* and *entB*. Phage 29 was propagated on strains COL and 592, and the sterile lysates were used to transduce strain 8325-4(ϕ 11) for resistance to tetracycline. The transduction frequency of the Tc^r marker from strain COL was 4.6×10^{-6} and that from strain 592 was 2.4×10^{-6} . As a control to rule out linkage of *entB*

with the Tc^r plasmid, 10 randomly selected 8325-4(ϕ 11) Tc^r *Mec*^r transductants from each donor were propagated in NP broth for 36 h and the spent medium was analyzed for SEB. Additionally, analysis of 25 \times concentrated supernatants from the Tc^r *Mec*^r transductants was performed. The data demonstrated that none of the transductants synthesized SEB whereas the donor strains COL and 592 produced 12.5 and 25 μ g/ml, respectively. Plasmid DNA analysis of the Tc^r *Mec*^r transductants by agarose gel electrophoresis of cleared lysate preparations from either donor showed that the selected clones contained only the 3.0-Mdal plasmid (Fig. 3, lanes B and C). These results demonstrate that the *entB* gene is not linked to the 3.0-Mdal Tc^r plasmid in either strain. These data are consistent with our earlier findings (17) in various *Mec*^r SEB⁺ isolates. Moreover, since the 3.0-Mdal Tc^r plasmid is the only extrachromosomal DNA species harbored by strain COL, the *entB* gene is chromosomal in this particular isolate.

To determine if *entB* from strains 592 and COL, as in strain DU-4916 (3, 4, 19), could be

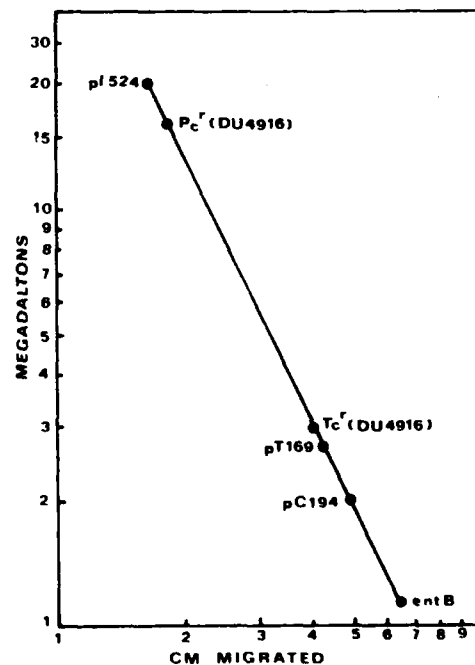


FIG. 2. Relative migration in 1% agarose gels of plasmid DNA from *S. aureus* DU-4916 to reference plasmids. The molecular weights of the reference plasmids (pI524, pT169, pC194) have been described elsewhere (11, 14, 17). The molecular weights of the DU-4916 plasmids were derived from the linear migration of the reference plasmids.

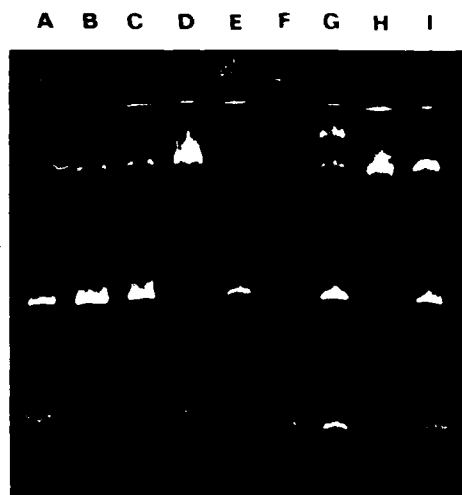


FIG. 3. Agarose gel electrophoresis of ethanol-precipitated cleared lysate DNA from SEB⁺ donors (*S. aureus* strains 592, COL, and DU-4916) and transductants. (A) 8325-4(ϕ 11) Tc^r Mec^r SEB⁺; (B) 8325-4(ϕ 11) Tc^r Mec^r SEB⁺; (C) 8325-4(ϕ 11) Tc^r Mec^r SEB⁺; (D) 8325-4(ϕ 11) Tc^r Mec^r SEB⁺; (E) electrophoretically purified 3-Mdal Tc^r plasmid from *S. aureus* COL; (F) electrophoretically purified 1.15-Mdal SEB plasmid from *S. aureus* DU-4916; (G) *S. aureus* 592; (H) *S. aureus* COL; (I) *S. aureus* DU-4916. The plasmid DNA profiles of the transductants in lanes A and B were obtained from transducing lysates obtained on *S. aureus* 592 whereas lanes C and D were obtained from phage propagated on *S. aureus* COL.

cotransduced with *mec* and *tc*, we selected for tetracycline-resistant clones of 8325-4(ϕ 11) and scored the transductants for the Mec^r phenotype (Table 3). Clones exhibiting both phenotypes, as determined by replica plating on agar medium containing both drugs, were analyzed for toxin production and plasmid DNA. When strain COL served as the donor, *mec* was cotransduced with *tc* at a frequency of 6% (12/200). All of the double transductants were SEB⁺ (5 to 12.5 μ g/ml). Agarose gel electrophoresis of cleared lysates from representative clones demonstrated only the presence of the 3.0-Mdal Tc^r plasmid. The plasmid profiles of the Tc^r Mec^r SEB⁺ and the Tc^r Mec^r SEB⁺ derivatives were therefore identical (Fig. 3, lanes C and D). When strain 592 served as the donor for *tc* (Table 3), the Mec^r phenotype was cotransduced at a frequency of 2% (4/200). In agreement with the results obtained with COL, all of the *mec* cotransductants were also SEB⁺. However, all of the Tc^r Mec^r SEB⁺ clones contained both the 3.0- and 1.15-Mdal plasmids, whereas Tc^r Mec^r

SEB⁺ derivatives contained only the 3.0-Mdal plasmid (Fig. 3, lanes A and B). These results obtained with strain 592 show that it is similar to strain DU-4916 (19). Additionally, the relatively high cotransduction of *mec* with *tc* that we have observed in both COL and 592 may reflect the transient genetic association of these two markers as proposed by Shalita et al. (19).

We have been able to transduce the *mec* determinant by direct selection (Table 3) into 8325-4(ϕ 11) from strains COL and 592 but at very low frequencies (2×10^{-9} to 5×10^{-9}). Although this transduction frequency would generally be considered within the expected frequency of mutation to the Mec^r phenotype, our laboratory and others have not observed mutation of strain 8325-4(ϕ 11) to the Mec^r phenotype (3, 6, 8, 20; W. M. Shafer and J. J. Iandolo, unpublished data). When the *mec* determinant was transduced into 8325-4(ϕ 11) from either 592 or COL, a 100% cotransduction frequency with *tc* and *entB* was observed. The Mec^r Tc^r SEB⁺ transductants obtained from phage propagated on COL and 592, as expected, contained the plasmid profile of the parental strains.

Transduction of methicillin resistance was shown by Cohen and Sweeney (2) to occur at high frequencies when the recipient contained the penicillinase plasmid p1524 and was lysogenized by the prophage ϕ 11. The functions provided by these two elements in facilitating *mec* transduction remain unknown. We wished to determine if the low level of *mec* transduction into strain 8325-4(ϕ 11) could be increased by the presence of p1524. Therefore, we constructed strain 8325-4(ϕ 11)(p1524) by transducing p1524 from its natural host, RN492, into 8325-4(ϕ 11). When strain 8325-4(ϕ 11)(p1524) was employed as the recipient, the frequency of *mec* transduction from strains COL, 592, and DU-4916 increased approximately 100-fold (Table 3). The Mec^r transductants were scored for Tc^r and SEB synthesis. Neither the Tc^r plasmid nor *entB* was cotransduced with *mec* when the chromosomal SEB strain COL served as the donor; each marker individually and jointly segregated with *mec* at low frequencies when transducing lysates from the plasmid SEB strains, DU-4916 and 592, were employed. Cotransduction analysis demonstrated that the *entB* gene was only transduced when the clones were resistant to both tetracycline and methicillin. All of the Mec^r Tc^r clones analyzed were SEB⁺.

Regardless of the donor strain employed, analysis of the plasmid DNA profile of the 8325-4(ϕ 11)(p1524) *mec* clones demonstrated only the presence of the p1524 plasmid (data not presented). The 8325-4(ϕ 11)(p1524) *mec* *tc* *entB*

TABLE 3 Cotransduction of *SEB* with *Mec*^r and *Tc*^r selection

Donor strain	Recipient	Selected phenotype ^a (no. clones/frequency)	Cotransduction
COL	8325-4(φ11)	Tc ^r 4600/4.6 × 10 ⁻⁶	Mec ^r (6%), SEB (6%)
	8325-4(φ11)	Mec ^r 4/2 × 10 ⁻⁶	Mec ^r and SEB (100%) ^b
	8325-4(φ11)(pI524)	Tc ^r 1300/1.3 × 10 ⁻⁶	Tc ^r (100%), SEB (100%)
	8325-4(φ11)(pI524)	Mec ^r 260/2.6 × 10 ⁻⁶	Tc ^r and SEB (100%) ^b
592	8325-4(φ11)	Tc ^r 2400/2.4 × 10 ⁻⁶	Mec ^r (0.923%), SEB (0.78%)
	8325-4(φ11)	Mec ^r 6/5 × 10 ⁻⁶	Mec ^r and SEB (85%)
	8325-4(φ11)(pI524)	Tc ^r 1100/1.1 × 10 ⁻⁶	Tc ^r (10%), SEB (0%)
	8325-4(φ11)(pI524)	Mec ^r 560/5.6 × 10 ⁻⁶	Mec ^r (2%), SEB (2%)
DU-4916	8325-4(φ11)(pI524)	Tc ^r 4500/4.5 × 10 ⁻⁶	Mec ^r (100%), SEB (100%)
	8325-4(φ11)(pI524)	Mec ^r 650/6.5 × 10 ⁻⁶	Tc ^r (100%), SEB (100%)

^a Frequencies are expressed as the number of transductants per plaque-forming unit.^b Expressed as the percentage of *Mec*^r *Tc*^r cotransductants that are *SEB*^r.

derivatives obtained from the 592 and DU-4916 transducing lysates were found to contain pI524, the 3.0-Mdal *Tc*^r plasmid, and pentB (data not presented).

To determine if the *Tc*^r plasmid was involved in the successful transduction of *mec* and *entB*, we also employed strain 8325-4(φ11)(pI524) as the recipient in *tc* transduction. The donor strains consisted of COL, 592, and DU-4916. Transduction of *tc* from strain COL occurred at a frequency of 1.3 × 10⁻⁶, and the *Mec*^r phenotype was cotransduced at a frequency of 0.92%. Several *Tc*^r *Mec*^r and *Tc*^r *Mec*^r transductants were propagated in 3% NP, and the spent media were analyzed for toxin. All of the *Tc*^r *Mec*^r derivatives were found to be *SEB*^r, whereas six of seven *Tc*^r *Mec*^r cotransductants were *SEB*^r. Plasmid DNA analysis of these clones revealed only the presence of pI524 and *Tc*^r plasmid (data not presented). When strains DU-4916 and 592 were the donors of *tc*, both *mec* and *entB* were cotransducible. The frequency of *mec* cotransduction using phage propagated on DU-4916 was 0.76 and 3.8% when 592 was the donor strain. *SEB* analysis of the *Tc*^r *Mec*^r and *Tc*^r *Mec*^r transductants obtained from both DU-4916 and 592 demonstrated that tetracycline selection facilitated the joint cotransduction of *mec* and *entB*.

In summary, the cumulative transduction results obtained demonstrate that *entB* is not physically linked to the *Tc*^r plasmid or closely

linked to the *mec* gene in any of the three strains analyzed. However, *SEB*^r transductants were apparent when the recipients were jointly cotransduced for both *mec* and *tc* but neither marker alone. Plasmid DNA analysis of *SEB*^r transductants also revealed that, in strain COL, the *entB* gene is chromosomal, whereas the determinant is linked to the pentB plasmid in strains 592 and DU-4916. A summary of the transduction results obtained in this study is provided in Table 3.

The involvement of the tetracycline plasmid in these manipulations led us to investigate whether a resident *Tc*^r plasmid was necessary for *entB* transduction. Specifically we wished to evaluate whether a resident *Tc*^r plasmid performs a role in establishing *entB* or *mec* into the chromosome. We transduced the *Tc*^r plasmid from DU-4916S (17) into 8325-4(φ11) and 8325-4(φ11)(pI524). Using these transductants as recipients, we transduced for *Mec*^r and screened for *SEB* production. Transduction of *mec* from COL was not observed when 8325-4(φ11) *tc* was the recipient but did occur when the pI524 derivative was used (frequency of 2.3 × 10⁻⁶). Twenty randomly picked 8325-4(φ11)(pI524) *tc mec* derivatives were propagated in broth and analyzed for *SEB*. All were *SEB*^r. These data indicate that a resident *Tc*^r plasmid does not enhance either *mec* or *entB* establishment. Rather, the data shown in Table 3 indicate that transmission of the *Tc*^r plasmid plays a critical

role in establishment of the *Mec^r SEB⁺ P⁺* phenotype. The *entB* genetic variability (i.e., plasmid versus chromosomal loci) and the fact that *mec* also behaves as a transposition element (20) suggests that the *Tc^r* plasmid may be involved as an intermediate vector of either or both genes. In the foregoing experiments, we have been unable to separate *entB* from *mec*. That is, we have never isolated an *SEB⁺* transductant that was not also *Mec^r*. However, the converse is possible; the majority of the *Mec^r* clones that have been isolated are *SEB⁺* (this study; 9, 19). These findings prompted us to examine the degree and manner of linkage of the *mec* and *entB* genes.

Based on a high frequency of coelimination, Dornbusch et al. (3, 4) suggested that *mec* and *entB* genes were linked. Because our transduction data were radically different unless associated with a *Tc^r* plasmid, we examined this question using two different elimination strategies. We employed culturing in ethidium bromide and a recent procedure for *mec* elimination outlined by Kuhl et al. (8) involving transduction of novobiocin resistance (*nov^r*) into *mec* strains.

Curing experiments were carried out by culturing strain COL in TSB containing 9×10^{-6} M ethidium bromide and 592 in TSB containing 4.5×10^{-6} M ethidium bromide. These concentrations were the minimal inhibitory concentrations for each isolate. After incubation at 37°C

for 36 h, 500 colonies from each isolate were analyzed for antibiotic resistance and SEB synthesis by replica plating onto TSA containing the desired antibiotic and NP antibody agar. When strain 592 was analyzed by this procedure, two classes of derivatives were obtained; *Pc⁺ Tc⁺ Mec^r SEB⁺* (5% elimination frequency) and *Pc⁺ Tc⁺ Mec^r SEB⁻* (0.80%) clones (Table 4). The former lacked only the 17.5-Mdal plasmid but synthesized SEB at the same level as the parental (25 µg/ml). Conversely, the latter lacked both the 3.0- and the 1.15-Mdal plasmids and did not produce SEB even when 25× concentrated culture supernatants were analyzed. When strain COL was propagated in ethidium bromide broth, the *Tc^r* and *Mec^r* phenotypes were not eliminated (500 surviving colonies were analyzed [Table 4]). We therefore analyzed the stability of the *entB* determinant by replica plating all colonies onto NP antibody agar plates. This procedure did not reveal any *SEB⁺* derivatives. As reported by others (3, 19), conventional elimination procedures strongly suggest linkage of these two genes among plasmid *SEB* producers. The chromosomal strains were remarkably stable and, to assess linkage in this group, we employed the following approach.

Kuhl et al. (8) recently demonstrated that the *mec* determinant is site specific and maps in the *pyr-his-nov-pur-mec* linkage group. Transduction revealed that, when *Mec^r Nov^r* recipients

TABLE 4. Plasmid DNA profile of antibiotic-sensitive and toxin-negative derivatives

Strain	Phenotype	Mol wt of plasmids ^a	Source
592	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i>	17.5, 3.0, 1.15	Wild type
592 A	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i> (5.0%, 24/500) ^b	3.0, 1.15	Ethidium bromide curing
592 B	<i>Pc⁺ Tc⁺, Mec^r, SEB⁺</i> (0.8%, 4/500)	17.5	Ethidium bromide curing
592 <i>nov^r</i>	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i> (100%, 500/500)	17.5, 3.0, 1.15	<i>Nov^r</i> transductant from ISP2
COL	<i>Tc⁺, Mec^r, SEB⁺</i>	3.0	Wild type
COL <i>nov^r</i>	<i>Tc⁺, Mec^r, SEB⁺</i> (96%, 288/300)	3.0	<i>Nov^r</i> transductant from ISP2
COL <i>nov^r</i>	<i>Tc⁺, Mec^r, SEB⁺</i> (4%, 11/300)	3.0	<i>Nov^r</i> transductant from ISP2
COL <i>nov^r</i>	<i>Tc⁺, Mec^r, SEB⁺</i> (0.3%, 1/300)	None	<i>Nov^r</i> transductant from ISP2
57-dk	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i>	17.5, 3.0	Wild type
57-dk A	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i>	3.0	Ethidium bromide curing
57-dk- <i>nov^r</i>	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i> (88%, 176/200)	17.5, 3.0	<i>Nov^r</i> transductant from ISP2
57-dk- <i>nov^r</i>	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i> (3.5%, 7/200)	17.5, 3.0	<i>Nov^r</i> transductant from ISP2
57-dk- <i>nov^r</i>	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i> (1.5%, 3/200)	17.5, 3.0	<i>Nov^r</i> transductant from ISP2
57-dk- <i>nov^r</i>	<i>Pc⁺, Tc⁺, Mec^r, SEB⁺</i> (7%, 14/200)	17.5, 3.0	<i>Nov^r</i> transductant from ISP2

^a Expressed in megadaltons as determined by agarose gel electrophoresis.

^b Reported as the frequency of isolation.

were rendered Nov⁺ by transduction, the Mec⁺ phenotype was lost at low frequencies (1 to 5%). To test whether methicillin resistance and SEB synthesis could be eliminated by this procedure, phage 29 was propagated on strain ISP2 and used to infect strains 592 and COL. Using strain COL as the recipient, *nov* was transduced at a frequency of 2×10^{-7} (Table 4). The Nov⁺ transductants were subsequently replica plated onto TSA, TSA-methicillin, and NP antibody agar. This procedure demonstrated a 4% (12/300) frequency of *mec* elimination. Of the 12 Mec⁺ clones, all were SEB⁺ and one derivative also lost the Tc^r phenotype. These derivatives were analyzed for plasmid DNA by agarose gel electrophoresis, and the Tc^r Mec⁺ SEB⁺ clones contained the 3.0-Mdal Tc^r plasmid whereas a single Tc^r Mec⁺ SEB⁺ clone lacked this 3.0-Mdal species. Therefore, except for one derivative, SEB⁺ clones of strain COL contain the same plasmid DNA profile as the parental. When strain 592 was used as the recipient, the *nov*⁺ gene was transduced at a frequency of 9×10^{-8} . However, all of the Nov⁺ transductants retained both the Mec⁺ and SEB⁺ phenotypes. A similar approach was employed with another chromosomal *entB* isolate, strain 57-dk. Approximately 12.0% of the Nov⁺ transductants were found to be Mec⁺ or SEB⁺, and in about half (5%) the *mec* and *entB* genes were independently eliminated (Table 4).

Both approaches at elimination of genes (chemical curing and *nov* transduction) have resulted in an inconclusive general model of chromosomal linkage for *entB* and *mec*. Chemical curing of the *entB* gene was only effective with plasmid SEB⁺ strains and indeed suggests linkage with *mec*. The use of *nov* transduction proved to be an adequate strategy for *entB* elimination in chromosomal strains, but different results were obtained with each strain. In strain COL the genes for methicillin resistance and SEB behave as though they are closely linked because they coeliminate totally. On the other hand, in strain 57-dk linkage is much less pronounced, with each gene being independently eliminated.

To examine this region of the chromosome in more detail, we have analyzed the *nov-pur-mec* linkage group as defined by Kuhl et al. (8) for the presence of the toxin gene. The strains employed in this investigation were Nov⁺ and Pur⁺. We transduced the *nov* gene via phage 29 from ISP2 to the SEB⁺ strains DU-4916, 592, COL, and 57-dk. The Nov⁺ transductants were subsequently scored for the Mec⁺ and SEB⁺ phenotypes to insure that these phenotypes had not been eliminated. Selected clones exhibiting the Nov⁺ Mec⁺ SEB⁺ phenotypes were infected with phage 29, and the resulting sterile lysates were

employed for *nov* transduction. Using strain 8325-4(φ11) as the recipient, the frequency of *nov* transduction ranged from 3×10^{-8} to 5×10^{-7} , depending on the donor strain (Table 5). Cotransduction of *mec* occurred at low frequencies (2 to 8%). All of the 8325-4(φ11) *nov* transductants, regardless of the donor strain, however, remained SEB⁺. Therefore, *entB* does not belong to the *pur-his-nov-pur-mec* linkage group and reinforces our contention that these two genes are not closely linked.

To further confirm the results of these transduction experiments, we have employed Mec⁺ selection in transformation experiments with chromosomal DNA prepared as described by Sjöström et al. (20). Using this DNA preparation, Sjöström et al. were able to obtain high frequencies of *mec* transformation but were unable to obtain Mec⁺ transformants when the plasmid-containing fraction was employed. In our hands, using *S. aureus* strain 8325-4(φ11) as the recipient and donor DNA prepared from strains DU-4916, 592, COL, and 57-dk, the frequency of *mec* transformation ranged from 2×10^{-7} to 9×10^{-7} . Mec⁺ clones were subsequently replicated to NP antibody agar to score for the toxin phenotype. All Mec⁺ clones, regardless of the source of the donor DNA, did not produce SEB and were devoid of plasmids as determined by agarose gel electrophoresis (Fig. 4). Regardless of the source of the donor DNA, the only ethidium bromide-staining material evident from the 8325-4(φ11) *mec* transformants was chromosomal DNA. Therefore, by this criterion it also appears that *mec* and *entB* are not closely linked genes.

DISCUSSION

A previous report from this laboratory (17) demonstrated that the gene responsible for SEB synthesis is capable of existing in either the plasmid or the chromosomal state. However, a degree of uncertainty still remains regarding the plasmid locus. The problem revolves around the unselectability of the *entB* gene. Analysis has been carried out by cotransfer of this relevant genotype with the determinant specifying meth-

TABLE 5. Transduction of *nov* from SEB-producing strains

Donor	Recipient	Transduction Frequency		
		Nov ⁺ ^a	Mec ⁺ ^b	SEB ⁺
COL- <i>nov</i>	8325-4(φ11)	4.57×10^{-7}	8%	0
57-dk- <i>nov</i>	8325-4(φ11)	3.62×10^{-7}	3%	0
592- <i>nov</i>	8325-4(φ11)	3×10^{-8}	2%	0
DU-4916- <i>nov</i>	8325-4(φ11)	5×10^{-8}	4%	0

^a Expressed as number per plaque-forming unit.

^b Expressed as cotransduction frequency.

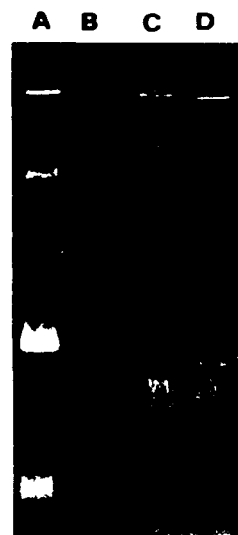


FIG. 4. Agarose gel electrophoresis of cleared lysate DNA extracted from *mec* transformants, *S. aureus* strains DU-4916, and 8325-4(ϕ 11). (A) *S. aureus* DU-4916; (B) *S. aureus* 8325-4(ϕ 11); (C) *S. aureus* 8325-4(ϕ 11) *mec*; (D) *S. aureus* 8325-4(ϕ 11) *mec*. The *mec* transformant in panel C was obtained from donor DNA of *S. aureus* DU-4916, whereas the *mec* transformant in panel D was obtained from donor DNA of *S. aureus* COL.

icillin resistance. Apparent genetic linkage has been reported between these two markers (3, 4), and although not 100% it is greater than one would predict for the random association of genes. Therefore, the SEB⁺ phenotype has always been determined in either plasmid (3, 4, 19) or chromosomal strains (this study) by screening among a background of methicillin-resistant isolates. Although this situation might suggest that the plasmid *entB* locus in particular is an artifact of the assay system, the complete (i.e., 100%) correlation of the plasmid with the SEB⁺ phenotype among such strains argues strongly for this configuration.

The purpose of this investigation was twofold. Firstly, we have been concerned with the physical disposition of the *entB* gene and have wished to confirm the existence of a plasmid responsible for SEB synthesis in a strain other than DU-4916. Secondly, we have been interested in detailing the linkage arrangement of *entB* with the tetracycline and methicillin resistance genes.

A survey of the extrachromosomal DNA profile of 15 *Mec*⁺ isolates demonstrated that only a minority of strains (37.5%) contain the 1.15-Mdal plasmid presently implicated in SEB synthesis. One of these isolates, *S. aureus* 592, was chosen for genetic examination, and the results

obtained were consistent with previous work in strain DU-4916 (19). Specifically, the loss of SEB synthesis due to elimination by culturing in the presence of ethidium bromide resulted in the concomitant loss of the 1.15-Mdal plasmid. Additionally, transduction of *entB*, via selection for methicillin and tetracycline resistance, into strain 8325-4(ϕ 11) or the pl524-containing derivative demonstrated that all SEB⁺ clones harbored the 1.15-Mdal plasmid. Although we do not presently have direct biochemical evidence to demonstrate that the structural gene is present on this plasmid, it is clear from both our results and Shalita et al. (19) that this plasmid is critically involved in the establishment of enterotoxigenesis.

Our data have also demonstrated that, in other *Mec*⁺ isolates, the *entB* gene can occupy a chromosomal locus. This was shown when the single plasmid harbored by strain COL failed to confer the toxin phenotype when transferred. Further evidence showing that the *entB* gene is maintained differently in strains COL and 592 was reflected by the phenotypic stability of SEB synthesis. As is characteristic of plasmid and chromosomal genes, *entB* was eliminated in strain 592 but not COL when cultured in the presence of ethidium bromide.

Since the initial examination of *mec* and *entB* in *S. aureus* DU-4916 by Dornbusch and Hallander (3), numerous studies have attempted to elucidate the genetic status of *mec*. The cumulative data have unambiguously shown that *mec* is chromosomal in most if not all isolates (6, 8, 20, 22). Transformation of *mec* has been shown by Sjöström et al. (20) to be independent of *recA* function(s) and has therefore been implicated as a potential translocatable element. Kuhl et al. (8) have established the chromosomal map site for *mec* as belonging in linkage group II on the staphylococcal chromosome and have suggested that, if *mec* is a translocatable element, insertion is site specific.

The rigorous examination of *mec* by other investigators (6, 8, 20, 22) has ignored the question of linkage with *entB*. Therefore, the original proposal of linkage made by Dornbusch et al. (4) has not been satisfactorily verified. Although SEB synthesis is characteristic of most *Mec*⁺ isolates (10), the results of this investigation demonstrate that *mec* and *entB* do not belong to the same linkage group when analyzed by transformation and transduction. However, when analyzed by transduction, both genes can be transduced at low frequencies with the Tc^r plasmid. The surprisingly high frequency (ca. 5×10^{-8} to 2×10^{-7}) with which this event occurs argues against a chance packaging of these physically unlinked genes by a transducing particle.

In fact, cotransduction of the *entB* gene, regardless of the plasmid or chromosomal nature of the determinant, was only obtained when the recipient was rendered resistant to both methicillin and tetracycline. Transduction of either resistance marker alone did not promote co-transfer of *entB*. Furthermore, this role does not appear to be unique to the Tc^r plasmid carried by any single strain, since identical results have been obtained with at least three different strains. This occurrence reinforces the contention of Shalita et al. (19) that all three genes may be transiently associated during transduction. The basis for this anomaly is not presently understood, although our observations suggest that, during transduction, the Tc^r plasmid performs an obligatory step in the cotransfer of the *mec* and *entB* genes. This seems likely because prior residence of the Tc^r plasmid in the recipient does not result in productive transduction of both *mec* and *entB*. In a dynamic sense such transient associations would initially involve *mec* and the Tc^r plasmid forming a genetic unit which may or may not include *entB*, and that, once established, the genes are positioned in the correct configuration. This latter event is underscored by the lack of any single recipient clone containing a single plasmid element possessing all three genes.

The results obtained in this and our previous investigation (17) of the genetic determinant responsible for SEB clearly demonstrate the complexity of analysis of the *entB* gene. Our findings of chromosomal and plasmid loci involved in toxin synthesis and the interrelationship with other genetic elements suggest that the *entB* gene may be capable of transposition activity. Work with recombination-deficient mutants is in progress and should resolve this question. Moreover, additional study of the association of *mec* and *entB* and the role the Tc^r plasmid may play in the establishment of these genes is also necessary to understand the system.

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Transduction of Staphylococcal Enterotoxin B Synthesis: Establishment of the Toxin Gene in a Recombination-Deficient Mutant†

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Cotransduction of enterotoxin B synthesis into a recombination deficient mutant only occurred when the donor contained the pEntB plasmid. Enterotoxin B from chromosomal genotypes could not be established in such hosts. These data suggest that the *entB* gene is not capable of high-frequency translocation.

In other communications (8, 9) we showed that the genetic determinant for staphylococcal enterotoxin B (*entB*) can be either plasmid borne or chromosomal. About one-third of the strains analyzed possessed the plasmid genotype, and all of these were methicillin resistant (*Mec*^r). The remaining two-thirds of the strains possessed chromosomal determinants of staphylococcal enterotoxin B (SEB) and were either *Mec*^r and *Mec*^s.

Genetic analysis showed that the *entB* gene could only be studied in those strains that were *Mec*^r. In fact, methicillin resistance and SEB cotransduced with high frequency (8-10), although they do not appear to be closely linked genes (9). Furthermore, the mobility of the *entB* gene from either plasmid or chromosomal donors seems dependent upon cotransfer of *mec* and a tetracycline resistance plasmid.

Methicillin resistance has already been suggested to be a translocatable genetic element (11). The strong association exhibited by *mec* and *entB* and the plasmid-chromosomal genetic duality exhibited by the enterotoxin determinant suggest that *entB* may also possess the ability to translocate. We have examined this possibility by employing a recombination-deficient (*recA1*) mutant as a recipient in the transduction of *entB*. This approach is based on observations from several laboratories (5, 6, 11, 12) that staphylococcal plasmids and high-frequency translocation elements (but not chromosomal genes) are readily established in such hosts.

We compared the ability to transfer the SEB phenotype from plasmid and chromosomal *entB* genes into both *rec*⁺ and *rec* recipients. The donor and recipient strains employed in this

study are listed in Table 1.

We employed strains 8325-4(ϕ11)(pI524) and 8325-4(ϕ11)(pI524) *his-7 recA1* as recipients for *mec* transduction because Cohen and Sweeney (2) showed that *mec* was transduced at high frequencies when the recipient strain contained a 20×10^6 -dalton (20-megadalton [Mdal]) penicillinase plasmid (pI524) and was lysogenized by the prophage ϕ11. Strain 8325-4(ϕ11)(pI524) *his-7 recA1* was constructed by transducing pI524 from strain 8325-4(pI524) into 8325-4(ϕ11) *his-7 recA1*. The *recA1* genotype was confirmed by the inability to induce ϕ11 (16) and the inability to serve as a recipient for a chromosomal gene (*nor*). The stringency of the *recA1* genotype was evident by the ability to be transduced for plasmid-associated tetracycline resistance (*Tc*^r) at a frequency of 10^{-6} and the inability to obtain novobiocin-resistant transductants (using ISP2 as the donor; frequency, $<10^{-10}$).

The preparation of phage 29 transducing lysates and transduction has been described elsewhere (8, 9). Tetracycline-resistant (*Tc*^r) transductants were selected on Trypticase soy agar plates containing 5 µg of tetracycline per ml and were screened for the phenotype by replicating onto agar plates containing 5 µg of methicillin per ml and 5% NaCl. The process was reversed for primary selection of *Mec*^r transductants. When *Mec*^r transductants were desired, the phage preparation was treated with ultraviolet light before infection in order to increase the transduction frequency (2). All *Mec*^r *Tc*^r clones were repurified on agar containing both drugs. Enterotoxin production by single colonies was determined immunologically by Elek plate analysis (1, 9) and Laurell immunoelectrophoresis (3, 8, 9) of 36-h culture supernatants. Plasmid deoxyribonucleic acid (DNA) analysis was performed as described by Shafer and Iandolo (9).

In Table 2 it can be seen that *Mec*^r *Tc*^r clones from DU-4916 were obtained when either *Mec*^r or *Tc*^r was initially selected. However, when

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strain COL was the donor of *Mec*⁺ and *Tc*⁺, the double transductants were only obtained when the initial selection was for *Tc*⁺. All *Mec*⁺ *Tc*⁺ clones were screened for SEB production. The results demonstrate that when DU-4916 (which hosts pEntB) served as the donor, toxin synthesis was cotransduced with *Mec*⁺ and *Tc*⁺ equally well into either the *recA1*⁺ or *recA1* recipient. With primary selection for *Mec*⁺, 30% of the *Tc*⁺ cotransductants were SEB⁺ in the *recA1* host, whereas 52% of the *recA1*⁺ cotransductants were SEB⁺. When *Tc*⁺ was used for primary selection with the *recA1* host, 100% of the *Mec*⁺ cotransductants were SEB⁺. The corresponding control showed 30% SEB⁺ *Tc*⁺ *Mec*⁺ cotransductants.

When strain COL (chromosomal *entB* genotype) served as the donor for *Mec*⁺ and *Tc*⁺, SEB⁺ transductants were only obtained when

the *recA1*⁺ recipient was employed. To determine whether the *Mec*⁺ *Tc*⁺ *recA1* transductants obtained from strain COL produced low levels of SEB which were not detected by Elek plate analysis, several clones were propagated in 3% NP broth for 36 h, and the culture supernatants were concentrated 50-fold by pervaporation (3). The concentrated samples were analyzed for SEB by immunoelectrophoresis and were found to be devoid of toxin.

Agarose gel electrophoresis of cleared lysates from donor strains, recipient strains, and transductants was performed to verify the plasmid profiles of each clone. The agarose gel profile of the donor strains (DU-4916 and COL), the recipient strain 8325-4(ϕ11)(p1524) *his-7 recA1*, and the transductants is presented in Fig. 1. The plasmid DNA profile of the parent strain DU-4916 is presented in lane A, and its SEB⁺ and SEB⁺ derivatives of 8325-4(ϕ11)(p1524) *his-7 recA1 mec tc* are presented in lanes E and F. Both clones were found to contain the resident p1524 plasmid and the transduced *Tc*⁺ plasmid. However, the SEB⁺, but not the SEB⁺, derivative also contained the 1.15-Mdal pEntB plasmid present in strain DU-4916. Similar gel profiles were obtained when the *recA1*⁺ transductants from strain DU-4916 were analyzed for the presence of plasmid DNA (data not presented).

The plasmid DNA profile of the transductants using strain COL as the donor of *mec* and *tc* was also analyzed. The 3-Mdal plasmid responsible for tetracycline resistance in strain COL (lane B) was resolved in all transductants resistant to tetracycline (lane D). Furthermore, SEB⁺

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype ^a	Source
DU-4916	<i>cd bla tc mec entB</i>	S. Cohen
COL	<i>tc mec entB</i>	B. Wilkinson
8325-4 (p1524)	<i>cad bla</i>	R. P. Novick
8325-4 (ϕ11)	ϕ11	P. A. Pattee
8325-4 (ϕ11) (p1524)	ϕ11 <i>bla cad</i>	This laboratory
8325-4 (ϕ11) <i>his-7 recA1</i>	ϕ11 <i>recA1</i>	R. P. Novick
8325-4 (ϕ11) (p1524) <i>his-7 recA1</i>	ϕ11 <i>bla cad recA1</i>	This laboratory
ISP2	8325 <i>nor</i>	P. A. Pattee

^a Abbreviations are as follows: *cd*, cadmium resistance; *bla*, beta-lactamase production; *tc*, tetracycline resistance; *mec*, methicillin resistance; *entB*, enterotoxin B; *nor*, novobiocin resistance; ϕ11, prophage ϕ11.

TABLE 2. Transduction studies using a recombination deficient mutant

Donor	Recipient	Selected phenotype ^a	Cotransduction	<i>Mec</i> / <i>Tc</i>	% SEB	Plasmid profile ^b	
						SEB ⁺	SEB
DU-4916	<i>recA1</i> ⁺	<i>Mec</i> ⁺ , 6.5 × 10 ⁻⁶ (650)	<i>Tc</i> ⁺ , 5% (33/650)	3.3 × 10 ⁻⁶	52 (17/33)	20/3/1.15	20/3
DU-4916	<i>recA1</i>	<i>Mec</i> ⁺ , 4.9 × 10 ⁻⁶ (490)	<i>Tc</i> ⁺ , 4.7% (23/490)	2.3 × 10 ⁻⁶	30.4 (7/23)	20/3/1.15	20/3
DU-4916	<i>recA1</i> ⁺	<i>Tc</i> ⁺ , 4.5 × 10 ⁻⁶ (4,500)	<i>Mec</i> ⁺ , 0.75% (34/4,500)	3.4 × 10 ⁻⁶	30 (10/34)	20/3/1.15	20/3
DU-4916	<i>recA1</i>	<i>Tc</i> ⁺ , 1.47 × 10 ⁻⁶ (971)	<i>Mec</i> ⁺ , 0.65% (6/971)	9.0 × 10 ⁻⁶	100 (6/6)	20/3/1.15	20/3
COL	<i>recA1</i> ⁺	<i>Mec</i> ⁺ , 2.6 × 10 ⁻⁶ (260)	<i>Tc</i> ⁺ , 0%		0		20
COL	<i>recA1</i>	<i>Mec</i> ⁺ , 6.9 × 10 ⁻⁶ (690)	<i>Tc</i> ⁺ , 0%		0		20
COL	<i>recA1</i> ⁺	<i>Tc</i> ⁺ , 1.3 × 10 ⁻⁶ (1,300)	<i>Mec</i> ⁺ , 0.92% (12/1,300)	1.2 × 10 ⁻⁶	83 (10/12)	20/3	20/3
COL	<i>recA1</i>	<i>Tc</i> ⁺ , 2.78 × 10 ⁻⁶ (2,780)	<i>Mec</i> ⁺ , 0.65% (18/2,780)	1.8 × 10 ⁻⁶	0 (0/100)		20/3

^a Frequencies are expressed as the number of transductants per plaque-forming unit. Parentheses indicate number of clones screened.

^b Expressed in megadaltons as determined by agarose gel electrophoresis. Numbers are three different molecular weights of the three plasmids under consideration.

^c Total number of *Mec*⁺ *Tc*⁺ transductants screened from at least three separate experiments.

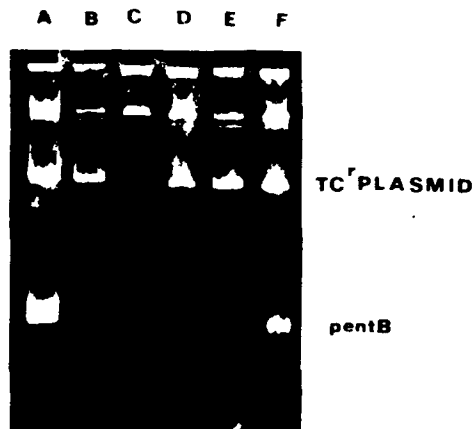


FIG. 1. Agarose gel electrophoresis of cleared lysate DNA. (A) *Staphylococcus aureus* DU-4916; (B) *S. aureus* COL; (C) *S. aureus* 8325-4(ϕ 11)(pI524) *his*-7 *recA*⁺; (D) 8325-4(ϕ 11)(pI524) *his*-7 *recA*⁺ *mec* *tc*; (E) 8325-4(ϕ 11)(pI524) *his*-7 *recA*⁺ *mec* *tc*; (F) 8325-4(ϕ 11)(pI524) *his*-7 *recA*⁺ *mec* *tc* *entB*. The transductant presented in (D) was obtained using *S. aureus* COL as the donor for *Mec*⁺ *Tc*⁺; *S. aureus* strain DU-4916 was the donor for the clones presented in (E) and (F). Details regarding the conditions of electrophoresis are presented in reference 7.

(*recA*⁺) and SEB⁺ (*recA*⁺ and *recA*⁺) transductants resistant to both methicillin and tetracycline had the same plasmid DNA profile. In other experiments, transductants resistant to tetracycline but not methicillin also harbored the 3-Mdal plasmid. A summary of the genetic and plasmid DNA results obtained is presented in Table 2.

The finding that *entB* from strain DU-4916 was established in both 8325-4(ϕ 11)(pI524) and the *recA*⁺ derivative confirms previous reports (9, 10) that the gene is plasmid borne in this strain. Additionally, the association of *entB* with the 1.15-Mdal plasmid is evident.

We recognize that translocation of chromosomal *entB* was not unequivocally examined because of the inability to select directly for SEB⁺ transductants. However, these experiments in the *recA*⁺ recipient confirm the lack of plasmid linkage in chromosomal strains and indicate that any potential independent translocation of *entB* must occur at a very low and presently undetectable frequency. That is, if the transduction frequency of 10^{-4} for *entB* noted in this work (from strain COL to the *recA*⁺ recipient; Table 2) is multiplied by the probability of translocation of other staphylococcal trans-

sons of about 10^{-4} per donor genome (7), then the combined probability of 10^{-12} is well below the limits of detection of this system. Indeed, this same argument was recently stated by Phillips and Novick (7) to explain the inability to detect translocation of Tn 551 in *recA*⁺ recipients by transduction.

In contrast, the ability to establish chromosomal methicillin resistance in the *recA*⁺ recipient suggests that this gene does translocate at high frequency. Further, this finding underscores the absence of linkage between *mec* and *entB*, which we reported earlier (9), even though such linkage seems evident in the wild type (Table 2).

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Plasmid-Chromosomal Transition of Genes Important in Staphylococcal Enterotoxin B Expression†

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Experiments were performed to further elucidate the genetic mechanisms underlying the synthesis of staphylococcal enterotoxin B (SEB). Our laboratory has previously shown that, in strains of *Staphylococcus aureus* which harbor a 1.15-megadalton plasmid (*pentB* or pSN2), the plasmid appears to be required for SEB synthesis; in other *S. aureus* strains, designated chromosomal SEB producers, this 1.15-megadalton plasmid is conspicuously absent. We report here that in both *Bacillus subtilis* minicells and a coupled translational assay, pSN2 codes for a polypeptide of 18,000 daltons. This product is not immunologically reactive with purified anti-SEB globulin. Nevertheless, pSN2 is necessary but not sufficient for SEB synthesis in strains which harbor the plasmid. Further, the data provide a reasonable link between plasmid-bearing and chromosomal SEB producers: transformational analysis indicates that both require functions specified (in plasmid-bearing strains) by pSN2 for SEB synthesis. The combined genetic and biochemical data suggest that pSN2 is not the reservoir for the SEB structural gene, but that the pSN2-specific functions required for SEB synthesis are regulatory in nature.

In recent years, the genetic basis for staphylococcal enterotoxin B (SEB) synthesis has been the subject of increasing attention. However, the genetic determinants required for SEB production remain unidentified. Earlier, Dornbusch et al. (3) studied SEB production in *Staphylococcus aureus* DU-4916, a methicillin-resistant clinical isolate. These authors concluded, upon the basis of cotransduction and coelimination data that the *Mec*^r and SEB markers might be physically linked on a small plasmid. Shalita et al. (15) examined this *Mec*^r SEB⁺ strain with respect to SEB production and concluded that a small plasmid, pSN2 (we [14] referred to this plasmid as *pentB*), was critical for enterotoxinogenesis. Previous studies reported by our laboratory (14) confirmed the correlation between enterotoxin B production and this small 1.15-megadalton (Mdal) plasmid in strain DU-4916 and a number of other *S. aureus* SEB producers. In another report (13), we also screened a number of *Mec*^r SEB⁺ isolates and found that the majority did not possess pSN2. In transduction experiments using these strains as donors, SEB⁺ transductants arose which never contained pSN2. Consequently, the question of plasmid involvement in SEB synthesis has not been altogether clear.

We have used transformation and protoplast fusion techniques to determine the involvement of pSN2 in SEB synthesis. Additionally, we have conducted experiments to clarify the protein products specified by pSN2 in *Bacillus subtilis* minicells and in an in vitro coupled system. The data suggest that functions specified by pSN2 are required in plasmid-bearing and in chromosomal SEB producers, and that pSN2 is capable of supporting SEB synthesis from a chromosomal or an extrachromosomal site. Upon the basis of the genetic, minicell, and in vitro experiments, we propose that the synthesis of enterotoxin B in *S. aureus* is dependent upon at least two unlinked genes. We further propose that pSN2 provides regulatory functions essential for SEB synthesis rather than serving as a reservoir for the SEB structural gene.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are described in Table 1.

Media and chemicals. Trypticase soy broth (BBL Microbiology Systems) was used for routine cultivation of *S. aureus* strains and *B. subtilis* 168 and was supplemented with thymine and methionine (100 µg/ml each) for *B. subtilis* CU403 *div IV B1* and its derivatives. Solid media consisted of Trypticase soy broth supplemented with 1.5% agar (Difco Laboratories). For toxin production and analysis, cells were grown in 3% N-Z amine-3% protein hydrolysate (NAK-PHP) (13) broth.

† Contribution no. 81-461-j, Division of Biology, Kansas Agricultural Experiment Station, Manhattan, KS 66506

TABLE 1. *Bacteria used in this study*

Strain	Derivation	Relevant phenotype ^a	Plasmid profile ^a	Source
<i>B. subtilis</i> 168 derivatives				
KSI500	<i>B. subtilis</i> 168		None	J. Urban
KSI501	Transformant of KSI500	Cm ^r	pC194	This study
KSI502	Transformant of KSI500	Cm ^r SEB	pC194, pSN2	This study
KSI503	Transformant of KSI500	Cm ^r Tc ^r SEB	pC194, pSN1, pSN2	This study
<i>B. subtilis</i> CU403 derivative derivatives				
KSI520	<i>B. subtilis</i> CU403 derivative		None	N. Mendelson
KSI521	Transformant of KSI520	Cm ^r	pC194	This study
KSI522	Transformant of KSI520	Cm ^r SEB	pC194, pSN2	This study
<i>S. aureus</i> strains				
RN450	RN450		None	
KSI400	RN450 fused with KSI522 micelles	Cm ^r	pC194, pSN2	This study
KSI390	DU-4916	Pc ^r Tc ^r Mec ^r SEB ^r	pSN1, pSN2, pSN3	S. Cohen
KSI391	DU-4916S	Pc ^r Tc ^r Mec ^r SEB ^r	pSN1	S. Cohen
KSI392	SEB derivative of KSI390	Pc ^r Tc ^r Mec ^r SEB ^r	pSN1, pSN2, pSN3	This study
KSI393	SEB derivative of KSI390	Pc ^r Tc ^r Mec ^r SEB ^r	pSN1, pSN3	This study
KSI109	S6	Cd ^r SEB ^r	Cd ^r plasmid	M. Bergdoll
KSI110	S6R	Cd ^r SEB ^r	Cd ^r plasmid	This study
KSI111	SEB ^r transformant of KSI110	Cd ^r SEB ^r	Cd ^r plasmid	This study

E. coli CSH63

^a Marker abbreviations: Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; Pc^r, penicillin resistance; Cd^r, cadmium nitrate resistance; Mec^r, methicillin resistance.

^b pC194 is a 2.0-Mdal plasmid specifying chloramphenicol resistance (5). pSN1 and pSN3 are 3.0-Mdal Tc^r and 17.5-Mdal Pc^r plasmids, respectively (14). pSN2 is a 1.15-Mdal cryptic plasmid (15). The Cd^r plasmid is a 17.5-Mdal species found in *S. aureus* KSI109, from which KSI110 and KSI111 were derived (13).

Biochemicals were generally purchased from Sigma Chemical Co., with the following exceptions: pyruvate kinase and proteinase K were from Boehringer-Mannheim; [2-³H]thymidine (specific activity, 17 Ci/mmol) was from Schwarz Mann; and [³⁵S]methionine (specific activity, 1,100 Ci/mmol) was from New England Nuclear. Highly purified, crystalline SEB was a gift from J. Metzger (U.S. Army MRDC, Fort Detrick, Md.). Antiserum directed against SEB was prepared in New Zealand white rabbits and a domestic goat.

DNA purification. Chromosomal deoxyribonucleic acid (DNA) for transformation was prepared as described by Sjoström et al. (17). Cleared lysates for preparation of plasmid DNA were made as described in a previous paper from this laboratory (14). Cesium chloride-ethidium bromide equilibrium buoyant density gradient centrifugation for purification of covalently closed circular DNA from cleared lysates was also performed as previously described by this laboratory (13). All DNA concentrations were determined by absorbance at 260 nm.

Electrophoretically purified plasmids were extracted from 1% preparative agarose gels which had been stained with ethidium bromide. The plasmid band was excised from the gel with a clean razor blade, minced, and forced through a syringe into a sterile 30-ml Corex centrifuge tube. A 2-ml sample of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 5.9) was added. Agarase was then added to the mixture at a final concentration of 0.5 mg/ml. The

tube was incubated at 37°C for 2 h, followed by holding the mixture overnight on ice. The particulate material was removed by centrifugation (43,500 × *g* for 30 min). The DNA was ethanol precipitated by the addition of NaC₂H₃O₂ to 0.3 M and 2 volumes of cold 95% ethanol. After 12 to 18 h at -20°C, the DNA was pelleted by centrifugation and dissolved in a small volume of TE buffer [10 mM tris(hydroxymethyl)aminomethane-1 mM ethylenediaminetetraacetic acid, pH 7.5]. This solution was phenol extracted three times with redistilled phenol saturated with TE buffer and then dialyzed for 24 h against two changes of 1 liter of TE buffer (13). The DNA was again ethanol precipitated, dissolved in TE buffer, and extracted three times with TE-saturated *n*-butanol to remove any residual ethidium bromide. The DNA was ethanol precipitated a third time and dissolved in a small volume of TE buffer before use.

For bulk purification of pSN2 DNA from *S. aureus* KSI390, an overnight culture was diluted 1:100 in fresh Trypticase soy broth containing 250 µg of deoxyadenosine per ml and grown at 37°C with shaking until the culture reached 5 Klett units (usually about 30 min). Plasmid DNA was then labeled with [2-³H]thymidine by adding label to the culture at a final concentration of 4 µCi/ml. Incubation was continued for an additional 4 h, and a cleared lysate was prepared from this culture. Equal aliquots of this cleared lysate were layered over 13-ml, 5 to 20% sucrose gradients in 0.1 × saline-sodium citrate (SSC); 1 × SSC is 0.15 M NaCl-

0.015 M sodium citrate, pH 7.0; approximately 350 μ g of DNA was layered over each gradient. The gradients were centrifuged at $200,000 \times g$ in a Beckman SW41 rotor for 10.5 h at 4°C. Fractions (0.2 ml) were collected from each gradient, and the position of each plasmid was determined by counting a small portion (usually 10 μ l) of each fraction. Appropriate fractions were pooled and analyzed by agarose gel electrophoresis to assess the purity of the plasmid preparation. Usually two successive sucrose gradients were necessary to prepare pSN2. Purity of all plasmid preparations was estimated by first photographing the ethidium bromide-stained agarose gel and then scanning the photographic negative in a Gilford model 240 spectrophotometer with the gel-scanning attachment. The densitometric tracing was used to estimate plasmid purity. Typically, pSN2 was greater than 97% pure by this method.

Transformations. Introduction of plasmid or chromosomal DNA into various *S. aureus* recipients has been described previously (14). Plasmid DNA was transformed into *B. subtilis* 168 (KSI500) or *B. subtilis* CU403 *div-IV B1* (KSI520) by the protoplast transformation procedure of Chang and Cohen (2). Plasmid-bearing minicells isolated from *B. subtilis* CU403 derivatives were fused with *S. aureus* recipients by the method of Stahl and Pattee (M. L. Stahl and P. A. Pattee, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, H32, p. 113) for fusion of *S. aureus* protoplasts.

Marker analysis. Antibiotic sensitivities were determined on Trypticase soy agar plates into which the appropriate antibiotic had been incorporated: chloramphenicol (5 and 15 μ g/ml) or tetracycline (5 μ g/ml). **Screening for the SEB⁺ phenotype** was performed on NAK-PHP plates into which 1 to 5% anti-SEB serum had been incorporated. Alternatively, SEB⁺ clones were identified by the immunological screening method of Ohman et al. (12). A precipitin line near a colony was considered presumptive evidence of SEB synthesis. Any presumptive SEB⁺ phenotype identified in this manner was confirmed by analysis of NAK-PHP culture supernatants by Ouchterlony double diffusion or by a nitrocellulose passive blot assay. The blot assay consisted of electrophoresis of concentrated culture supernatants in a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferring the electrophoretically separated proteins to a sheet of nitrocellulose, and identifying SEB by staining the blot with ¹²⁵I-labeled affinity-purified anti-SEB immunoglobulin. The details of the blot assay and affinity-purified antibody preparation will be presented elsewhere (R. K. Tweten and J. J. Iandolo, manuscript in preparation).

Minicell assays. Minicells were purified from KSI520 and its derivatives by the sucrose gradient method of Shivakumar et al. (16). Plasmid-specific proteins were labeled with [³⁵S]methionine (100 μ Ci/ml), also as described by Shivakumar et al., except that the nonradioactive methionine concentration in the incubation medium was reduced 10-fold. This modification provided enhanced incorporation of the ³⁵S label. Minicell extracts were analyzed by SDS-polyacrylamide gel electrophoresis on a 17.5% gel (7). The gel was impregnated with 2,5-diphenyloxazole in dimethylsulfoxide (1), and labeled proteins were visu-

alized by fluorography (1). Kodak XRP X-ray film was used with Cronex Lightning-Plus enhancing screens, and the film was exposed at -70°C for 24 to 36 h.

Coupled transcription-translation assays. Cell-free assays for plasmid-specific proteins using an *E. coli* S-30 extract were performed as described by Miller (9), except that the reaction mix was prepared using 2.5 mM rather than 25 mM methionine. After 60-min of labeling with [³⁵S]methionine (10 μ Ci/ml), the incubation mixture (100 μ l) was precipitated with 1 ml of 20% trichloroacetic acid and boiled for 15 min. The precipitate was pelleted in an Eppendorf microfuge, suspended in 1 ml of distilled water by sonication, and washed twice with distilled water. The final pellet was dissolved in a small volume (usually 25 μ l) of 0.04 M sodium phosphate buffer (pH 7.2) containing 7% SDS, 18% 2-mercaptoethanol, 10% glycerol, and bromophenol blue tracking dye. This mixture was subjected to electrophoresis on a 17.5% SDS-polyacrylamide gel, and the plasmid-specific proteins were visualized by fluorography.

Ethidium bromide curing experiments. For curing of plasmids by ethidium bromide treatment, cells were inoculated into flasks containing concentrations of ethidium bromide ranging from 4.5×10^{-6} M to 1.8×10^{-5} M in 25 ml of Trypticase soy broth. These cultures were incubated in the dark at 37°C with agitation for 24 to 48 h. The cultures containing the highest concentration of ethidium bromide in which bacteria grew were used to plate onto nonselective Trypticase soy agar. Colonies from this medium were replica plated to selective and antibody agars and scored for loss of phenotypic characters.

Electrophoretic analysis. Electrophoresis of proteins under reducing and denaturing conditions was performed as described by Laemmli (7). Agarose gel electrophoresis of plasmid DNA was performed as described previously (14).

RESULTS

Since many staphylococcal plasmids are efficiently expressed in *B. subtilis* (2, 5, 16), we felt that placing pSN2 in this heterogenous host would effectively isolate the putative enterotoxin gene against an innocuous genetic background. The transformation was accomplished by mixing buoyant density gradient-purified bulk plasmids from *S. aureus* KSI390 with pC194 (from *S. aureus* RN2425) and transforming protoplasts of KSI500 by the protoplast procedure of Chang and Cohen (2). The inability to select directly for the SEB⁺ phenotype led us to screen for cotransformants with pC194, a 2.0-Mdal Cm^r staphylococcal plasmid, known to be expressed in KSI500 (2). Cleared lysates of three of the Cm^r *B. subtilis* transformants, KSI501, KSI502, and KSI503, are shown in Fig. 1A (lanes 2, 3, and 4). Each derivative contains pC194, and KSI502 and KSI503 also contain pSN2. Additionally, KSI503 also harbors pSN1, the 3.0-Mdal Tc^r plasmid. Neither KSI502 nor KSI503 produced extracellular SEB, and SEB was not

found intracellularly (data not shown). These data implied that, if the enterotoxin gene resided on pSN2, it was not expressed in *B. subtilis*.

To assess the expression of pSN2 in *B. subtilis*, we utilized the minicell-producing strain KSI520. To generate minicells employed in this study we protoplast transformed this strain with a mixture of electrophoretically purified pSN2 and pC194. Two clones were isolated, KSI521, containing pC194, and KSI522, containing both pC194 and pSN2 (Fig. 1B, lanes 2 and 3). As in the previous experiment, Ouchterlony analysis showed that the *B. subtilis* transformant KSI522 failed to produce SEB either extracellularly or intracellularly. Minicells were isolated from each of these derivatives, and plasmid-specific proteins were labeled with [³⁵S]methionine as specific by Shivakumar et al. (16). In these incubations chloramphenicol was added at a concentration of 0.25 µg/ml to induce the production of C-1 protein of pC194 (16). After a 30-min labeling period the minicells were collected by centrifu-

gation and protoplasts were generated in 25% sucrose and lysozyme. Electrophoresis of minicell extracts treated with SDS and 2-mercaptoethanol was performed on a 17.5% SDS-polyacrylamide gel, and the [³⁵S]-labeled proteins were visualized by fluorography. Typical results are shown in Fig. 2. The large (22,300-dalton) chloramphenicol-inducible, C-1 protein specified by pC194 is shown in lanes A and B. A second protein of 18,000 daltons (lane C) appears to be pSN2 specific, since it is present only in minicell extracts isolated from KSI522. In other experiments we immunoprecipitated minicell extracts of both KSI521 and KSI522 by a double antibody procedure with affinity-purified horse anti-SEB immunoglobulin and goat anti-horse serum. No labeled immunoreactive material appeared either by directly counting the immunoprecipitate or on analysis of these precipitates by 17.5% SDS-polyacrylamide gel electrophoresis. These findings are consistent with Ouchterlony data indicating that KSI522 did not produce SEB.

In vitro products specified by pSN2. This laboratory has previously reported the molecular mass of pSN2 to be 1.15 Mdal (14). Assuming no overlapping genes, pSN2 should code for a maximum of about 63,900 daltons of protein. However, in the minicell assays we only accounted for 18,000 daltons of coding capacity for pSN2. This amounted to about 28% of the total

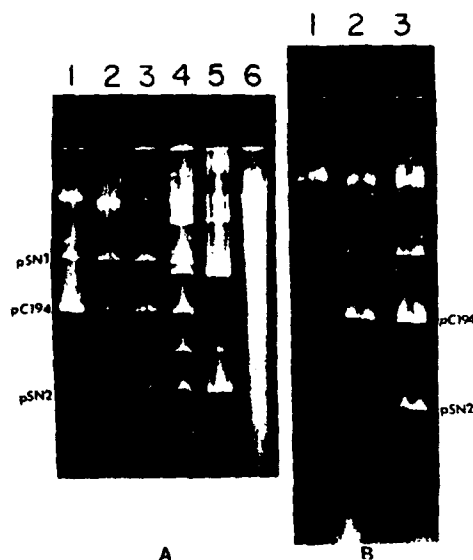


FIG. 1. Agarose gel electrophoresis of cleared lysate DNA. The conditions for electrophoresis in 1% agarose gels were as described previously (14). Panel A shows cleared lysate DNA from the following strains: (lane 1) RN2425, containing pC194; (lane 2) KSI501, containing pC194; (lane 3) KSI502, containing pC194 and pSN2; (lane 4) KSI503, containing pC194, pSN1, and pSN2; (lane 5) KSI390, containing pSN1, pSN2, and pSN3; (lane 6) KSI500, showing no plasmid DNA. Panel B shows cleared lysate DNA from the following strains: (lane 1) KSI520, containing no plasmids; (lane 2) KSI521, containing only pC194; (lane 3) KSI522, containing pC194 and pSN2.

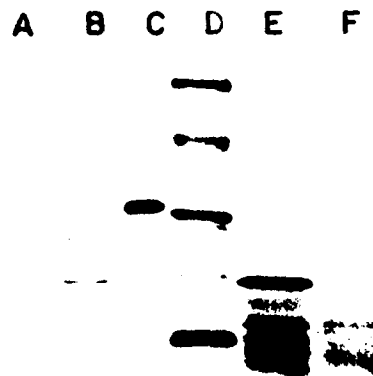


FIG. 2. Analysis of pSN2-specified protein products by 17.5% SDS-polyacrylamide gel electrophoresis. Lanes: A, [³⁵S]methionine-labeled proteins from KSI521 minicells containing pC194; B, [³⁵S]methionine-labeled proteins from KSI522 minicells containing both pC194 and pSN2; C, [¹²⁵I]-labeled SEB; D, [¹⁴C]-labeled molecular mass markers cytochrome c (12,400 daltons), chymotrypsin (25,000 daltons), ovalbumin (45,000 daltons), and bovine serum albumin (65,000 daltons); E, [³⁵S]methionine-labeled proteins from an *E. coli* S-30 extract programmed with 5 µg of pSN2; F, endogenous activity in *E. coli* S-30 extract.

capacity of the plasmid, clearly leaving enough capacity available for the enterotoxin B gene. (The mature toxin has a molecular mass of 28,366 daltons [6].) The remainder of pSN2 could still contain the SEB structural gene, although unexpressed in *B. subtilis* minicells. To assess whether pSN2 codes for any additional peptides, we employed a coupled transcription-translation assay using an *Escherichia coli* S-30 extract (9). However, after visualizing plasmid-specific [³⁵S]methionine-labeled proteins by fluorography of a 17.5% SDS-polyacrylamide gel electrophoresis, we again observed only the single 18,000-dalton protein (Fig. 2, lane E) as being clearly pSN2 specific. Immunoprecipitation also failed to show any immunoreactive material.

Transfer of pSN2 into *S. aureus* RN450.

The minicell and in vitro products of pSN2 observed in the experiments above suggested that, if pSN2 is the reservoir for the SEB structural gene, it must require factors specific to the staphylococcal protein synthetic apparatus for expression of the SEB⁺ phenotype. In our hands, an in vitro coupled assay with a staphylococcal extract similar to that described above for *E. coli* gave poor or variable results. Consequently, we transferred pSN2 to *S. aureus* RN450, which does not contain any extrachromosomal elements or any demonstrable lysogens (10). To do this we fused isolated KSI522 minicells (containing pC194 and pSN2) to RN450 cells by the protoplast fusion technique of Stahl and Pattee (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, H32, p. 113). This consisted of mixing protoplasts of both donor and recipient in a medium containing dimethyl sulfoxide and polyethylene glycol. After a short incubation, the mixture was plated into a nonselective protoplast regeneration medium. Surviving colonies were replica-plated onto chloramphenicol agar to screen for the residence of pC194. Any Cm^r isolates were then immunologically tested for SEB production, and agarose gel electrophoresis of minilysates was used to check for the presence of an autonomous pSN2 plasmid. A number of Cm^r clones were isolated, all of which contain pSN2 as well as pC194 (Fig. 3A, lane 7). However, none of these Cm^r cells were able to synthesize extracellular SEB when assayed by gel diffusion or nitrocellulose blot assay of culture supernatants (Fig. 3B, lane 8).

Requirement of pSN2 for SEB synthesis in *S. aureus* KSI390. Although many authors have clearly associated pSN2 with the elaboration of SEB (3, 14, 15), most SEB⁺ strains do not carry the plasmid and are able to produce the enterotoxin (13, 14). This observation, in addition to the data above, underscored the need to unambiguously demonstrate a plasmid-spe-

cific requirement for SEB synthesis. To demonstrate this requirement, we chose first to cure pSN2 from KSI390 by using ethidium bromide.

The results of this experiment are shown in Table 2. When KSI390 was cultured in the presence of 4.5×10^{-6} M ethidium bromide at 37°C and the survivors were plated on NAK-PHP agar, an unexpectedly high number (224 of 230, or 97.4%) of the cells screened at random, became SEB⁺. Fifteen of these SEB⁺ derivatives were screened for plasmid residence by a minilysate procedure; all contained pSN2. This strikingly high rate of segregation of SEB⁺ cells led us to question the stability of the SEB⁺ phenotype in cells not exposed to conditions which normally cure plasmids. This was particularly important since these SEB⁺ cells all seemed to contain pSN2. When KSI390 was grown at 37°C in the absence of ethidium bromide, 289 of 578 (50%) colonies screened had spontaneously lost the ability to produce SEB. All of the spontaneous segregants which we checked by minilysates contained pSN2. For comparative purposes, we checked the spontaneous rate at which the SEB⁺ phenotype appears in KSI109 and found no instability. In fact, none of 1,058 colonies of strain KSI109 grown overnight at 37°C spontaneously segregated the SEB⁺ phenotype.

At present we can offer no explanation for this high rate of segregation of the SEB⁺ phenotype in KSI390. However, these results can be interpreted as indicating that (i) pSN2 is not required for SEB synthesis in *S. aureus* KSI390 since all of the SEB⁺ clones examined still harbored pSN2, or (ii) pSN2 is required for SEB synthesis, but that elaboration of the enterotoxin is dependent upon a second element that is highly unstable in KSI390. We favor the latter explanation, based upon the data collected by other authors and the following experiments.

According to Novick et al. (11), certain small staphylococcal plasmids can be cured at a relatively high rate by plating protoplasts of a plasmid-bearing *S. aureus* strain onto a nonselective protoplast regeneration medium. After allowing regenerant colonies to arise, one can screen for loss of a particular plasmid by an appropriate method. Presumably this plasmid curing is the result of alterations in the cell membrane which either impair plasmid segregation during cell division or interfere with plasmid replication. Using this rather benign treatment, we were able to cure pSN2 from KSI390. The derivative, KSI393 (which still contained pSN1 and pSN3) (Fig. 3A, lane 4), remained Pc^r Tc^r Mec^r, but became SEB⁻. Purified minicells from KSI522 (containing pC194 and pSN2) were then used to make "mini-protoplasts" which were fused to protoplasts of KSI393. After regeneration, the

TABLE 2. Segregation of the SEB phenotype

Strain	Treatment	No. of colonies screened	No. of colonies SEB	Plasmid profile of SEB ⁺ cells
KSI390	4.5×10^{-6} M ethidium bromide	230	224 (97.4%)	pSN1, pSN2, pSN3
KSI390	None	578	289 (50%)	pSN1, pSN2, pSN3
KSI109	None	1,058	0	NA ^a

^a NA, Not applicable; KSI109 is a chromosomal SEB producer.

survivors were replica plated onto selective agar and scored for chloramphenicol-resistant clones. A number of Cm^r colonies were found to produce SEB by both Ouchterlony gel diffusion analysis and nitrocellulose blot (Fig. 3B, lane 6). A control experiment fusing KSI521 minicells with protoplasts of KSI393 produced a large number of Cm^r clones, none of which produced SEB.

Minicellates of the Cm^r SEB⁺ isolates (from the KSI522 minicell/KSI393 fusion) and the Cm^r SEB⁺ isolates (from both fusion experiments) failed to show plasmid bands corresponding to pC194 or to pSN2 (Fig. 3A, lane 5). These results indicate that fusion of the KSI393 recipient protoplasts with KSI522 minicells introduced pC194 and pSN2 into the recipient in a state which allowed immediate integration into the host chromosome. Chromosomal integration has recently been reported for pC194 (8), but not for pSN2. Since SEB⁺ cells did arise when recipient cells were fused with KSI522 minicells (containing pC194 and pSN2), but not when KSI521 minicells (containing pC194 only) were used as the plasmid donor, we concluded that pSN2 is an essential element for enterotoxigenesis in strain KSI390. Introduction of the plasmids into the recipient in these experiments apparently occurred in such a way that pC194 and pSN2 could only rarely establish autonomy. These results indicated that if pSN2 is unable to replicate in an autonomous fashion it must be capable of integrating into the host chromosome to support SEB synthesis. This behavior may be due to cell membrane alterations introduced during the protoplasting and fusion procedure that are similar to the membrane changes suggested by Novick et al. (11) to be responsible for protoplast curing. Importantly, if removed from the genetic background of KSI390, the plasmid alone is incapable of supporting SEB synthesis but seems to be a requisite for SEB production in strain KSI390. These experiments also show the requirement of a second element (probably located in the chromosome) for enterotoxin synthesis in KSI390.

Requirement of plasmid sequence for SEB synthesis in KSI109. The results above indicated that pSN2 is required for toxin production in *S. aureus* KSI390. These results also

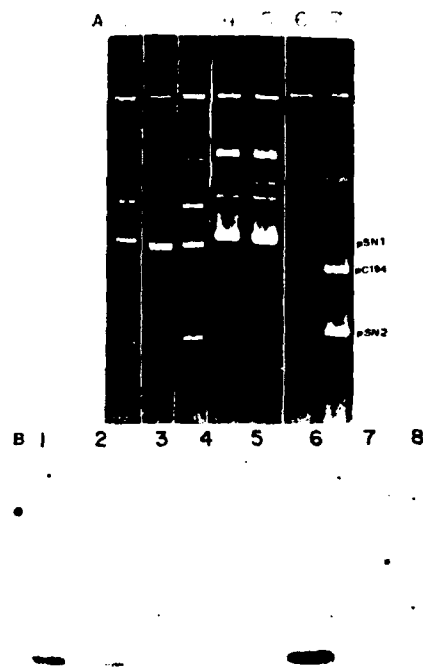


FIG. 3. A, Agarose gel electrophoresis of cleared lysate DNA. Lanes: 1, KSI390; 2, KSI391; 3, KSI392; 4, KSI393; 5, Cm^r isolate of KSI393 fused with minicells from KSI522; 6, *S. aureus* RN450; 7, KSI400. B, Nitrocellulose blot assay of culture supernatants, electrophoresed by 17.5% SDS-polyacrylamide gel electrophoresis and stained with affinity-purified, ¹²⁵I-labeled anti-SEB; Lane 1 contains 1 µg of SEB. Lanes 2 through 8 are culture supernatants from: 2, KSI390; 3, KSI391; 4, KSI392; 5, KSI393; 6, KSI393 fused with minicells from KSI522; 7, RN450; 8, KSI400.

implied that pSN2 sequences could support enterotoxin B synthesis from a chromosomal site and pointed directly to the requirement of pSN2 functions for SEB synthesis in chromosomal SEB producers. To test this hypothesis, we conducted a series of transformations between two KSI390 derivatives and strain KSI110, a spon-

taneous SEB derivative of KSI109, *S. aureus* KSI110 fails to produce any material which cross-reacts with anti-SEB immunoglobulin. We chose transformation rather than the minicell fusion technique used in other experiments because KSI109 and KSI110 are resistant to chloramphenicol (data not shown), presumably from a chromosomal locus. The obvious difficulty in transformations of this nature is that one cannot select directly for the SEB⁺ phenotype. Further, the screen employed for picking SEB⁺ transformants (agar medium containing antiserum) is often complicated by protein A interactions. Therefore, each presumptive SEB⁺ isolate was confirmed by Ouchterlony gel diffusion analysis of culture supernatants.

The results of these transformations are shown in Table 3. Cleared lysate DNA from KSI392 was used to transform KSI110. This KSI390 derivative was isolated from the ethidium bromide curing experiment described earlier. KSI392 is an SEB⁺ clone which is Pc⁺ Tc⁺ Mec⁺ and which harbors all three of the plasmids (pSN1, pSN2, and pSN3) that the parent strain KSI390 possesses. When the KSI110 transformants were analyzed, 24 SEB⁺ clones (1%) were isolated from 2,661 colonies screened. These KSI110 transformants, although able to synthesize SEB, did not contain pSN2 autonomously. When the reverse transformation was performed (that is, when chromosomal DNA from KSI110 was used to transform KSI392), 14 of 1,475 colonies (0.9%) were SEB⁺ and contained the same plasmid profile as the untransformed recipient. It should be pointed out that this latter transformation frequency is unusually high for staphylococcal chromosomal genes. Although we cannot adequately explain this, it may represent the movement of a transposition-like activity as recently proposed by Mallonee, Glatz, and Pattee for *entA* (Proc. Amer. Soc. Microbiol. p. 197, 1981). In both of these experiments no spontaneous SEB⁺ revertants were detected when untransformed recipients were plated directly to antibody agar.

In a similar set of experiments, KSI391 (14), a Pc⁺ Tc⁺ Mec⁺ SEB⁻ cell which lacks pSN2, was used as both donor and recipient in transformations with strain KSI110. When KSI391

cleared lysate DNA was used to transform KSI110, none of the 2,670 transformants screened became SEB⁺. To insure that the recipient KSI110 culture was competent for transformation, we also screened for recipients containing pSN1 by selecting for tetracycline resistance. The Tc⁺ phenotype appeared in the transformed population at a frequency of about 1% (32 of 2,670 clones). Minilysates of these transformants showed the presence of the 3.0-Mdal Tc⁺ plasmid, pSN1 (data not shown). This 1% transformation frequency is the same as the frequency obtained for the SEB⁺ clones in the KSI392/KSI110 transformations above and showed competence for transformation was present even though no SEB⁺ clones were isolated. When KSI110 chromosomal DNA was used to transform KSI391 none of the 1892 recipient clones screened were SEB⁺.

We conclude that functions specified by pSN2 in plasmid bearing strains are required for SEB synthesis in KSI110⁺. Additionally, this strain must harbor pSN2 (or pSN2-like) sequences within the host chromosome. This is consistent with the observation that fusions between KSI393 protoplasts and minicells containing pSN2 produce SEB⁺ cells which appear to have pSN2 chromosomally integrated.

DISCUSSION

The purpose of this study was to clarify the role of pSN2 in SEB synthesis. This was necessary since this laboratory has shown (14) that the majority of SEB⁺ isolates examined produce SEB without harboring this 1.15-Mdal plasmid, yet pSN2 appears essential for enterotoxigenesis in those strains carrying the replicon. We approached the resolution of this apparent controversy by directly evaluating the phenotypic expression and translation products of pSN2 in the heterogenetic background provided by *B. subtilis*. However, when we placed pSN2 into *B. subtilis* SEB production was not observed in the transformant clones. This might be expected if pSN2 were not fully expressed in *B. subtilis* or, alternatively, if the plasmid did not contain the SEB structural gene. However, on the basis of the minicell and the *in vitro* assays we concluded that the plasmid was expressed in these systems, but that SEB was not produced. In a recent review by Elwell and Shipley (4), the authors cite unpublished data from Novick's group which suggests that pSN2 specified a 20,000-dalton protein and an 11,000-dalton protein in experiments similar to ours. We assume that the 20,000-dalton protein seen in their experiments is that identified by us as having a molecular mass of 18,000 daltons. In some *in vitro* translation experiments we have also seen a second

TABLE 3. Transformations between KSI390 derivatives and KSI110

Donor	Recipient	No. of colonies screened	No. of colonies SEB ⁺
KSI392	KSI110	2,661	27
KSI391	KSI110	2,670	0
KSI110	KSI392	1,475	14
KSI110	KSI391	1,892	0

polypeptide of about 11,000 daltons that appears to be pSN2 specific. We have been unable to consistently reproduce these results and are consequently unsure about the origin of this 11,000-dalton protein. Additionally, unpublished nucleotide sequence data of Novick (also cited by Elwell and Shipley [4]) indicate that codons corresponding to the amino acid sequence of SEB (6) do not reside on the majority (approximately two-thirds) of pSN2. If the plasmid requires a function provided by any staphylococcal cell for SEB synthesis, then KSI400 (the RN450 derivative containing pSN2) should have been enterotoxigenic. However, KSI400 remained SEB⁻. Therefore, it seems likely that the structural gene for SEB does not reside on pSN2 but that expression of the SEB⁺ phenotype requires a second unlinked gene (the structural SEB gene) not present in *S. aureus* RN450.

An obvious alternative to this interpretation is that this cryptic plasmid is not involved in SEB synthesis. In attempting to cure pSN2 from KSI390 with ethidium bromide, we found a spontaneous segregation of this strain to the SEB⁻ phenotype. Notwithstanding the ethidium bromide treatment, the SEB⁻ phenotypic segregation occurred in the absence of any demonstrable effect on the intracellular residence of pSN2. These SEB⁻ KSI390 segregants display a phenotype similar to KSI400. Both strains contain pSN2, yet are SEB⁻. The high rate of spontaneous segregation of SEB⁻ we have seen is startlingly different than the curing observed by Dornbusch et al. (3) of Mec⁺ and SEB⁺ phenotypes in this same strain. These authors observed a coelimination of the two markers (12.5% of acriflavine-treated cells). Neither the Mec⁺ nor the SEB⁺ phenotype was spontaneously lost. The spontaneous SEB⁻ derivatives we have isolated continue to be resistant to methicillin (data not shown).

Since we have shown that KSI390 segregates the SEB⁻ phenotype at a high rate, one might predict that KSI393 would become SEB⁻. This strain, although lacking pSN2, could be SEB⁻ due to the fact that it was a spontaneous SEB⁻ segregant. However, when minicells containing pSN2 were fused with KSI393, the recipients often became SEB⁺. It is difficult to explain how this could occur without concluding that pSN2 is required for enterotoxigenesis in strain KSI390. The fact that many Cm^r SEB⁻ colonies were isolated in this experiment can be explained as the result of two processes. First, if all minicells did not contain both pC194 and pSN2 one would not expect a complete correspondence between the Cm^r and SEB⁺ phenotypes. Second and more important, SEB⁻ cells could arise if instability in the SEB⁺ phenotype in KSI390 is

due to a second element required for enterotoxigenesis. If this segregation occurred in the absence of pSN2, that cell would remain SEB⁻ even after introduction of the plasmid into the cell.

Our data indicate that pSN2 is required for SEB synthesis in chromosomal SEB producers as well as in plasmid-bearing strains. This was initially inferred from the fact that KSI393 Cm^r SEB⁺ recipients in minicell fusion experiments contained neither pC194 nor pSN2 as autonomous replicons. Both elements became chromosomally incorporated. Originally, pSN2 could replicate autonomously in strain KSI390. One might suggest that the lack of autonomy displayed by the plasmids was an artifact of the protoplast fusion technique. However, this explanation is difficult to reconcile with the fact that a similarly constructed strain, KSI400, contains autonomous pC194 and pSN2 replicons.

In accordance with the suggestion that pSN2 could support SEB synthesis from a chromosomal site in KSI393, we found that an SEB⁻ KSI390 derivative containing pSN2 (KSI392) was able to complement a chromosomal SEB⁻ derivative to produce SEB. In these experiments we detected no autonomous plasmids in the recipient SEB⁺ cells which would correspond to pSN2. Since this plasmid did not appear in the recipient, we infer that a pSN2 element must be capable of integration into the recipient chromosome to support SEB synthesis. It would be difficult to support this contention without hybridization data, except that we have shown via the minicell fusion experiment that pSN2 is capable of integrating in the KSI390 chromosome in order to support SEB synthesis. Because only two species of DNA were contained in the minicells, no other conclusions seem reasonable. We are at present conducting hybridization experiments using the Southern technique (18) to identify the chromosomal site of pSN2 integration in strain KSI110 and to study the manner in which integration occurs.

What is the role of pSN2 in SEB synthesis? We propose that pSN2 regulates SEB synthesis by affecting the expression of the SEB structural gene present on the chromosome of certain *S. aureus* strains. This function (or functions) is expressed whether the plasmid remains an autonomous replicon or becomes chromosomal. Any discussion of the nature of the regulation of SEB synthesis by pSN2 would be highly speculative at the present time.

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THE STAPHYLOCOCCAL ENTEROTOXINS – A GENETIC OVERVIEW¹

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INTRODUCTION

The enterotoxins produced by the Gram positive pathogenic bacterium, *Staphylococcus aureus* are a group of extracellular proteins (Bergdoll 1972, Bergdoll *et al.* 1974) which are responsible for the clinical symptomology of staphylococcal food poisoning. Although many cases of staphylococcal food poisoning probably go unreported, epidemiological data reveals that these enterotoxins account for over 25% (Bergdoll 1972) of all reported food poisoning incidents. While the immediate manifestations of enterotoxin food poisoning are obvious to the clinician, any additional roles that these proteins play during infection remain unknown. However, enterotoxigenic strains have been isolated from patients with chronic osteomyelitis, pseudomembranous enterocolitis and scalded skin disease.

Research efforts to date have been primarily concerned with enterotoxin purification, detection, and the regulation of toxin synthesis. While these studies have contributed a considerable amount of information regarding enterotoxin biochemistry and synthesis, they have not advanced understanding of the molecular genetics of toxin synthesis. Consequently, there exists a dearth of information regarding the genetics of enterotoxin synthesis. Such information could reveal molecular interrelationships of the various enterotoxins, the transmissibility of the enterotoxigenic phenotype, and linkage relationships with genes responsible for antibiotic resistance or in the production of proteins involved in pathogenesis.

The present study was undertaken in order to clarify some aspects

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of the molecular genetics of enterotoxin synthesis. Although six distinct enterotoxins have been purified and enterotoxins A (SEA) and D (SED) are most commonly associated with food poisoning outbreaks; enterotoxin B (SEB) was chosen for genetic analysis because previous work by others (Bergdoll 1972) suggested that the SEB structural gene was extrachromosomal and was not linked to genes responsible for antibiotic resistance. This in turn, could conceivably provide insights which would facilitate the genetic analysis of the other enterotoxins.

GENETICS OF SEB

The genetic analysis of enterotoxin B production initially proved difficult to accomplish because of the inability to select directly from the SEB⁺ phenotype. This problem seemed to be alleviated when Dornbusch *et al.* (1969, 1973) analyzed SEB production in the methicillin-resistant (Mec^r) clinical isolate *Staphylococcus aureus* strain DU-4916 (KSI 390). Table 1 is a list of the major pertinent *S. aureus* strains mentioned in this report. The remaining strains are described in Tables 2 and 3. In transduction and elimination experiments these authors demonstrated an association between the Mec^r and SEB⁺ phenotypes. This association suggested physical linkage between the Mec^r and SEB genes on a plasmid. Transduction of the Mec^r phenotype into a methicillin-sensitive (Mec^s) recipient nearly always resulted in co-transduction of the SEB⁺ phenotype. Additionally, when these authors also treated this same strain with acriflavine, an agent known to induce plasmid loss, Mec^s derivatives always lost the capacity to produce SEB.

Although the association between the Mec^r and SEB⁺ characters is unclear at this point, it is now obvious that they do not co-exist on any stable plasmid species. However, other studies have shown that SEB synthesis is indeed associated with a particular class of plasmid found in a variety of SEB⁺ isolates. Shalita *et al.* (1977) examined the Dornbusch strain, DU-4916 (KSI 390), and found that the ability to produce SEB was associated with a small plasmid, whose molecular weight was estimated at 0.75×10^6 . This plasmid has since been designated pSN2 and is shown as the peak of fraction 23 in Fig. 1A.

Our laboratory confirmed the association between pSN2 and enterotoxigenicity in *S. aureus* DU-4916. The data presented in Fig. 2 demonstrate that pSN2 must be present in certain strains for the expression of SEB. The plasmid profiles of the strains shown in

Table 1. Bacteria used in this study

Strain	Derivation	Relevant Phenotype ¹	Plasmid Profile	Source
<i>Bacillus subtilis</i> 168 derivatives:				
KSI500	<i>B. subtilis</i> 168 ind	—	none	J. Urban
KSI501	transformant of KSI500	Cm ^r	pC194	this study
KSI502	transformant of KSI500	Cm ^r SEB	pC194, pSN2	this study
KSI503	transformant of KSI500	Cm ^r Tc ^r SEB	pC194, pSN1, pSN2	this study
<i>Bacillus subtilis</i> CU403 div-IVB1 derivatives:				
KSI520	<i>B. subtilis</i> CU403 div-IVB1	—	none	N. Mendelson
KSI521	transformant of KSI520	Cm ^r	pC194	this study
KSI522	transformant of KSI520	Cm ^r SEB	pC194, pSN2	this study
<i>Staphylococcus aureus</i> strains				
RN450	RN450	—	none	—
KSI400	RN450 fused with KSI522 minicells	CM ^r	pC194, pSN2	this study
KSI390	DU-4916	Pc ^r Tc ^r Mec ^r SEB ²	pSN1, pSN2, pSN3	S. Cohen
KSI391	DU-4916S	Pc ^r Tc ^r Mec ^r SEB ²	pSN1	S. Cohen
KSI392	SEB derivative of KSI390	Pc ^r Tc ^r Mec ^r SEB ²	pSN1, pSN2, pSN3	this study
KSI393	SEB derivative of KSI390	Pc ^r Tc ^r Mec ^r SEB ²	pSN1, pSN3	this study
KSI110	S6R	Cd ^r SEB ²	Cd ^r plasmid	M. Bergdoll
KSI111	SEB ² transformant of DSI110	Cd ^r SEB ²	Cd ^r plasmid	this study
<i>Escherichia coli</i> CSH63				

¹Marker abbreviations are: Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; Pc^r, penicillin resistance; Cd^r, resistance to cadmium nitrate; SEB, staphylococcal enterotoxin B; Mec^r, methicillin resistance.

²pC194 is a 2.0 Mdal plasmid specifying chloramphenicol resistance. pSN1 and pSN3 are 3.0 Mdal Tc^r and 17.5 Mdal Pc^r plasmids, respectively. pSN2 is a 1.15 Mdal cryptic plasmid. The Cd^r plasmid is a 17.5 Mdal species found in *S. aureus* S6, from which KSI110 and KSI111 were derived.

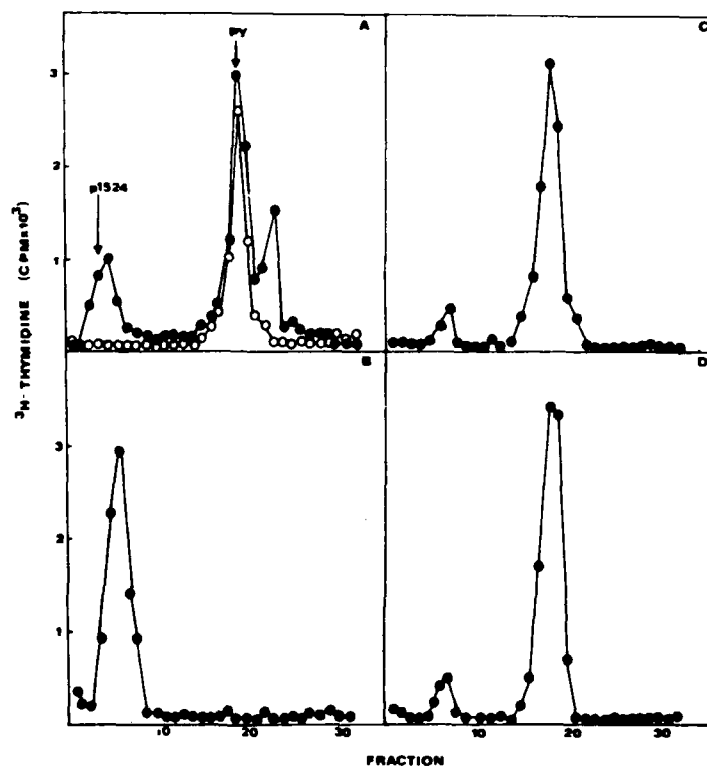


FIG. 1. DIRECT NEUTRAL SUCROSE GRADIENTS OF CLEARED LYSATES OF *S. AUREUS* (A) closed circles, strain DU-4916; open circles, strain KSI391; (B) strain S6; (C) strain 277; (D) strain 279. In A, the position of the marker DNA's (p1524-plasmid fraction 4 and polyoma virus-fraction 19) is indicated. Cleared lysates were centrifuged through 5-20% neutral sucrose in 1X SSC-0.05M tris(hydroxymethyl) aminomethane, pH 7.9 in an SW50.1 rotor for 180 min at 45,000 rpm.

lanes D and E show that when the donor (lane C) contained an autonomous pSN2 plasmid, SEB was only expressed in those transductants which also carried the plasmid as an independent replicon. Furthermore, we extended this observation (Shafer and Iandolo 1979) by showing that a significant number (37.5%) of the *Mec^R* SEB⁺ isolates examined (Table 2) contained a plasmid with the same molecular weight (1.5×10^6) as pSN2. Transductional analysis using these plasmid-bearing strains as donors was similar to that



FIG. 2. DEPENDENCE OF SEB PRODUCTION UPON THE PRESENCE OF pSN2

(A) electrophoretically purified pSN2; (B) cleared lysate DNA from the SEB⁻ recipient strain 8325-4(0-11); (C) cleared lysate DNA from the SEB⁺ donor strain DU-4916; (D) cleared lysate DNA from an SEB⁺ transductant of 8325-4(0-11); (E) cleared lysate DNA from an SEB⁻ transductant of 8325-4(0-11). Transduction was mediated by phage 29 grown on *S. aureus* DU-4916. Note the presence of pSN2 in the SEB⁺ strain (D) and its absence in the SEB⁻ strain (E).

mentioned above and clearly implicated this plasmid specie in SEB synthesis in these strains, as well as in *S. aureus* DU-4916.

In the majority of Mec^R SEB⁺ strains examined (62.5%), however, we were unable to find any plasmid DNA associated with SEB synthesis (Table 2). Rather, these isolates appeared to produce SEB from a chromosomal site. Additionally, those Mec^S SEB⁺ isolates which our laboratory examined also seemed to be "chromosomal" rather than "plasmid-associated" SEB producers. Genetic analysis

Table 2. Plasmid DNA profile of *Mec^s* SEB⁺ strains^a

Strain	Molecular Weight of Plasmids ^a	SEB Synthesis ^a
DU-4916	17.5 (Pc ^b), 3 (Tc ^b), 1.15 (SEB ^c)	50.0
592	17.5 (Pc ^b), 3 (Tc ^b), 1.15 (SEB ^c)	25.0
Kasanjian	22.4, 3.1	30.0
57-dk	17.5 (Pc ^b), 3.0 (Tc ^b)	23.0
5814R ^d	22.4 (Pc ^b), 1.8 (Cm ^b)	1.5
Meuse	17.5, 3.0	22.0
Japan	20, 3	23.0
Dumas	20, 3, 1.15	21.6
5106R	20, 3	16.7
7074R	20, 3, 1.15	11.9
5619	20, 3, 1.15	14.3
69129	20, 3, 1.15	15.5
COL	3 (Tc ^b)	12.5
456-33	20	18.0
5205-R	—	3.4
639-45I	20	10.9

^aExpressed in Megadaltons as determined by agarose gel electrophoresis^aExpressed as μ g/ml as determined by Laurell immunoelectrophoresis^aAll strains except for DU-4916 were supplied by B. Wilkinson^aA *Mec^s* SEB⁺ derivative (5814S), obtained from B. Wilkinson, was found to contain an identical plasmid profile^aBrackets indicate identified plasmid phenotypes. Unanalyzed plasmids were presumed to code for phenotypes appropriate to their size class

of these latter 2 groups is summarized in Table 3. None of the *Mec^s* strains examined (with the exception of KSI391—a cured derivative of DU-4916) showed any plasmid influence on SEB production and none contained a plasmid similar to pSN2. Figure 1 is typical of a neutral sucrose gradient of the resident plasmids carried in these organisms. Panel A (closed circle) shows strain DU-4916 which contains a prominent peak (fraction 23) corresponding to pSN2. The open circles are from an SEB⁺ derivative of strain DU-4916 (KSI391) and clearly lacks a pSN2 peak. Panels B, C and D are from various *Mec^s* SEB⁺ strains and they also lack pSN2 but carry other plasmids of larger size that are unassociated with SEB production. This last observation, that the majority of SEB⁺ strains examined do not contain a pSN2-like plasmid, could be explained in several ways. The plasmid, serving as the reservoir of the SEB structural gene, might be capable of existing as an autonomous replicon or as a chromo-

Table 3. Marker analysis

Strain	Mec ^r	SEB ^r	Plasmid Profile	
			Size (Mdal)	Relevant Phenotype ^b
S6	—	+	17.5	Cd ^r
S6Cd ^r	—	+	None	—
FRI 277	—	+	15	Pc ^r Cd ^r
			3	Tc ^r
FRI 279	—	+	15	Pc ^r Cd ^r
			3	Tc ^r
FRI 277 Pc ^r Cd ^r	—	+	3	Tc ^r
8325-4(Ø11)	—	—	None	—
8325-4(Ø11)(Cd ^r)	—	—	17.5	Cd ^r
8325-4(Ø11)(Tc ^r)	—	—	3	Tc ^r
DU-4916 (KSI390)	+	+	17.5	Pc ^r Cd ^r
			3	Tc ^r
			1.2	SEB ^r
DU-4916S (KSI391)	—	—	3	Tc ^r
COL	+	+	3	Tc ^r
57-dk	+	+	17.5	Pc ^r Cd ^r
			3	Tc ^r
5814R	+	+	22.4	Pc ^r Cd ^r
			1.8	Cm ^r

^aSEB produced in a 36 h 3% NAK-PHP broth culture

^bMec^r, methicillin resistant

These strains are various genetic derivatives. KSI391: Mec^r, Pc^r, SEB^r, Tc^r derivative of DU-4916 (S. Cohen). 8325-4(Ø11) Cd^r: obtained by transducing the Cd^r plasmid from S6. 8325-4(Ø11) Tc^r: obtained by transforming 8325-4(Ø11) with the Tc^r plasmid by FRI277. FRI277 Pc^r: obtained by eliminating Pc^r plasmid from FRI277. S6Cd^r: obtained by eliminating Cd^r plasmid from S6.

somally integrated specie. Alternatively, the plasmid might not be involved in enterotoxin synthesis. The association seen by many laboratories between pSN2 and SEB synthesis could merely be fortuitous. A third, albeit unlikely, possibility would be that plasmid-specific functions are required by "plasmid-bearing" SEB producers for SEB synthesis and that these functions are not required by so-called "chromosomal" SEB producers.

We addressed the question of a plasmid requirement for SEB synthesis by a variety of approaches (Dyer and Iandolo 1981). The first of these was to assess whether the plasmid could confer enterotoxigenicity upon an unrelated host. Since *Bacillus subtilis* has proven

to be an effective alternate host for a variety of staphylococcal plasmids (Gryczan *et al.* 1978, Chang and Cohen 1979, Shivakumar *et al.* 1979), we felt that transferring pSN2 from *S. aureus* strain DU-4916 to *B. subtilis* 168 would effectively isolate the presumed toxin gene against a relatively innocuous genetic background. However, when we placed pSN2 into *B. subtilis* 168 (see Fig. 3A), none of the recipients were able to produce SEB. In these experiments we selected for recipients co-transformed with pC194, a 2.0 Mdal staphylococcal plasmid specifying chloramphenicol resistance. This plasmid is known to be faithfully expressed in *B. subtilis* 168 (Gryczan *et al.* 1978, Chang and Cohen 1979), and served as an internal control for plasmid expression.

Since the *B. subtilis* 168 transformants that harbored pSN2 were not capable of elaborating SEB, we were faced with a variety of alternative explanations. Two alternatives were considered: (1) pSN2 might not be expressed in the *B. subtilis* host. This seemed unlikely, since both pC194 and pSN1 (see Fig. 3A) were faithfully

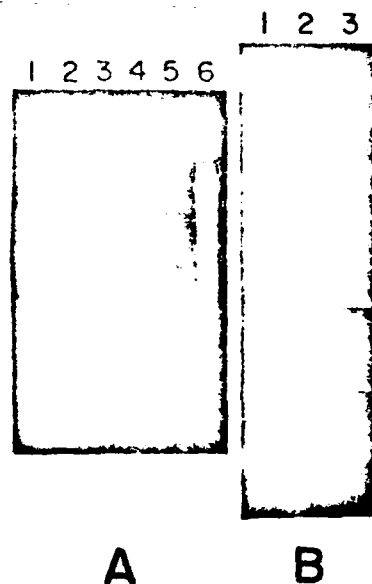


FIG. 3. PANEL A: CLEARED LYSATE DNA FROM

(1) *S. aureus* RN2425, containing pC194; (2) *B. subtilis* KSI501, containing pC194; (3) *B. subtilis* KSI502 containing pC194 and pSN2; (4) *B. subtilis* KSI503 containing pC194, pSN1 and pSN2; (5) *S. aureus* DU-4916 containing pSN1, pSN2 and pSN3; (6) *B. subtilis* 168 showing no plasmid DNA. Panel B: Cleared lysate DNA from: (1) KSI520, containing no plasmids; (2) KSI521, containing only pC194; (3) KSI522, containing pC194 and pSN2.

expressed (KSI502 is both Cm^R and Tc^R ; pSN1 is the 3.0 Mdal Tc^R plasmid from strain DU-4916). Consequently there seemed no barrier to expression of pSN2; (2) pSN2 might be expressed in the *B. subtilis* 168 transformants in exactly the same fashion that the plasmid is expressed in the *S. aureus* host, but pSN2 does not contain the SEB structural gene. To assess the expression of pSN2 in the *B. subtilis* host we transferred purified pSN2 into *B. subtilis* CU403 divIV-B1, a minicell-producing strain. Again, we selected recipients co-transformed with pC194 (see Fig. 3B). Strain KSI522, like the *B. subtilis* 168 transformants containing pSN2 that we originally isolated, did not produce any detectable enterotoxin B.

We isolated minicells from KSI521 (containing pC194) and from KSI522 (containing both pC194 and pSN2) and labelled plasmid-specific proteins with ^{35}S -methionine using the conditions specified by Shivakumar *et al.* (1979). When analyzed by SDS-polyacrylamide gel electrophoresis, extracts from KSI521 minicells (containing pC194) showed the presence of a single 22,300 dalton protein (Fig. 4, lane A). This was the C-1 protein previously demonstrated by Shivakumar *et al.* (1979) to be pC194-specific. Minicell extracts from KSI522 (containing pC194 and pSN2) contained two plasmid-specific proteins (Fig. 4, lane B), the 22,300 dalton C-1 protein of



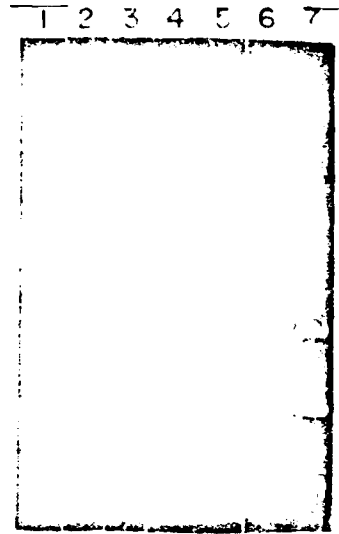
FIG. 4. ANALYSIS OF pSN2 SPECIFIED PROTEIN PRODUCTS BY 17.5% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

(A) ^{35}S -methionine labelled proteins from KSI521 minicells containing pC194; (B) ^{35}S -methionine labelled proteins from KSI522 minicells containing pC194 and pSN2; (C) ^{125}I labelled SEB; (D) ^{14}C -labelled molecular weight markers: cytochrome C, 12,400 dal; chymotrypsin, 25,000 dal; ovalbumin, 45,000 dal; bovine serum albumin, 65,000 dal; (E) ^{35}S -methionine labelled proteins from an *E. coli* S-30 extract programmed with 5 μg pSN2; (F) endogenous activity in *E. coli* S-30 extract.

pC194 and a second protein of 18,000 daltons which was pSN2-specific. Not only was this second protein 10,000 daltons smaller than the mature SEB molecule (28,366 daltons (Huang and Bergdoll 1970), see Fig. 4, lane C), but it did not react with affinity-purified anti-SEB immunoglobulin (Dyer and Iandolo 1981). We also placed pSN2 into an *in vitro* coupled transcription-translation system charged with an *Escherichia coli* S-30 extract (Miller 1972), and found that pSN2 specified again only an 18,000 dalton protein (Fig. 4, lane E). A recent review by Elwell and Shipley (1980) cites unpublished data from Novick's group which suggests that pSN2 specified a 20,000 dalton and an 11,000 dalton protein in experiments similar to ours. We assume that the 20,000 dalton protein seen in their experiments is that identified by us as having a molecular weight of 18,000 daltons. In some *in vitro* translation experiments we have also seen a second polypeptide of about 11,000 daltons that appears to be pSN2-specific. We have been unable to consistently demonstrate that this is a second pSN2-specific peptide and are consequently unsure of its origin.

We concluded that pSN2 was at least partially expressed in *B. subtilis* but was not capable of coding for SEB in this heterogenetic host. Based on these assays, we still could not distinguish whether the plasmid actually contained the SEB structural gene. Since we had only accounted for 28% of the maximum coding capacity of pSN2 (assuming no over-lapping genes), the plasmid might possess the SEB structural gene although unexpressed in these systems. This could be explained simply enough if pSN2 requires some factor peculiar to the staphylococcal protein synthetic apparatus for expression of the SEB structural gene. In our laboratory, an *in vitro* coupled assay system using a staphylococcal extract similar to the *E. coli* extract employed earlier gave poor or variable results. However, we reasoned that, if the SEB structural gene resided on pSN2 and merely required a staphylococcal host for expression, we should be able to transfer pSN2 from the *B. subtilis* host back to an SEB⁻ *S. aureus* recipient and regain enterotoxigenicity. We accomplished this transfer by fusing protoplasts of *S. aureus* RN450 (a strain which does not harbor any plasmid or demonstrable lysogens (Novick and Bouanchaud 1971)) with protoplasts of purified minicells from KSI522 (containing pC194 and pSN2). A number of Cm^r recipients were isolated, all of which contained pSN2 (see Fig. 5) but were still unable to produce SEB. These data increased our suspicions that pSN2 did not contain the SEB structural gene.

This is consistent with unpublished nucleotide sequence data from Novick's laboratory (cited in Elwell and Shipley 1980) which

FIG. 5. CLEARED LYSATE DNA FROM *S. AUREUS* STRAINS

(1) KSI390; (2) KSI391; (3) KSI392; (4) KSI393; (5) Cm^R SEB⁺ isolate of KSI393 fused with minicells of *B. subtilis* KSI522; (6) RN450; (7) KSI400.

indicated that codons corresponding to the amino acid sequence (Huang and Bergdoll 1970) of SEB did not reside on the majority (approximately 2/3) of pSN2. These observations also raised the possibility that pSN2 was uninvolved in SEB synthesis. Therefore we re-examined the involvement of pSN2 in SEB synthesis in strain DU-4916. We cured pSN2 from strain DU-4916 using the protoplast curing technique of Novick *et al.* (1980) and the resulting derivative, KSI393 (see Fig. 5) indeed had lost the capacity to produce SEB. When we re-introduced pSN2 into KSI393 by fusing KSI522 minicells with KSI393 protoplasts, the resulting Cm^R recipients were often SEB⁺. Since the appearance of both the Cm^R and SEB⁺ phenotypes in KSI393 occurred only when we used minicells containing pC194 and pSN2 (but not minicells containing only pC194) in fusion experiments, we concluded that pSN2 is required for SEB synthesis in strain DU-4916. Although critically required for SEB synthesis in strain DU-4916 (and by inference, in other plasmid-bearing SEB⁺ strains), we do not believe that the plasmid is the reservoir of the SEB structural gene. This conclusion is based upon the partial nucleotide sequence determined in Novick's laboratory, and our inability to demonstrate any SEB-coding capability in pSN2. Clearly,

the expression of the SEB⁺ phenotype is dependent upon the interaction of two physically unlinked genetic elements.

A second important observation was made in the experiments where we fused KSI393 protoplasts with minicells containing pC194 and pSN2. When we checked the plasmid profiles of the KSI393 recipients which became Cm^r SEB⁺, we found that these organisms did not contain autonomous pC194 or pSN2 replicons (Fig. 5). The obvious conclusion was that both plasmids became chromosomally integrated, a behavior reported for pC194 (Martin *et al.* 1981) but not for pSN2. In essence, what happened is that we converted a "plasmid-bearing" SEB producer (strain DU-4916) into a "chromosomal" SEB producer by removing pSN2 from DU-4916 and re-introducing it in such a way that chromosomal integration became the preferred manner for the cell to retain the incoming plasmid. How the switch was made to allow the plasmid to integrate rather than to remain autonomous is not clear at present. However, the integration of pSN2 into the host chromosome seen in these Cm^r SEB⁺ KSI393 recipients directly suggested that the distinction between "plasmid-bearing" and "chromosomal" SEB producers is quite artificial: both require pSN2-specified functions for SEB synthesis, the difference merely being the final disposition of the resident pSN2 element.

This line of reasoning predicted that we should be able to convert an SEB⁻ derivative of a chromosomal SEB producer to the SEB⁺ phenotype, if the SEB⁻ trait is due to the loss of pSN2-specific functions. Consequently, we undertook a series of transformations using two SEB⁻ DU-4916 derivatives. One derivative, KSI391 (Fig. 5), lacks pSN2, while a second, KSI392, contains pSN2 but lacks the second element critical for enterotoxigenesis (presumably, the SEB structural gene). Cleared lysate DNA was used to transform strain KSI110, a spontaneous SEB⁻ derivative of strain S6. Strain S6 is a typical chromosomal SEB producer (Shafer and Iandolo 1978). We obtained SEB⁺ cells when strain KSI110 was crossed with KSI392 (containing pSN2, see Table 4), but not when KSI391 (which does not contain pSN2) was used. In these experiments, the SEB⁺ derivatives of strain KSI110 did not contain an autonomous pSN2 element. This is an important observation for two reasons. Firstly, it indicates that strain S6 probably contains pSN2 inserted at a chromosomal site and that pSN2-specific functions (absent in KSI110) are required for SEB synthesis in this naturally occurring "chromosomal" SEB⁺ producing isolate. Secondly, the fact that pSN2 integrated in these KSI110 recipients suggests that integration is a function of the host cell and is independent of the method used to

Table 4. Transformations between DU4916 derivatives and KSI110

Donor	Recipient	Colonies Screened	SEB ⁺
KSI392	KSI110	2661	27
KSI391	KSI110	2670	0
KSI110	KSI392	1475	14
KSI110	KSI391	1892	0

introduce the plasmid into the recipient cell. This is important, since the conditions we used to force pSN2 integration into the KSI393 chromosome were the same that we originally used to cure pSN2 from DU-4916. The conditions that were used to transfer pSN2 into strain KSI110, on the other hand, would not be expected to unduly impair plasmid replication, since these transformation conditions do not induce the major cell membrane alterations that the protoplast fusion technique probably does (Novick *et al.* 1980).

One important question concerning the integration of pSN2 into the *S. aureus* chromosome is the location of the integrated plasmid. Recently, Pattee and Glatz (1980) attempted to transform the *entB*⁺ phenotype in strain S6 and *S. aureus* C234. However since these determinants were apparently located outside the three linkage groups of known staphylococcal genes (see Pattee and Neveln 1975 for details of these mapping procedures), they were unable to convert a set of multiply-marked auxotrophic derivatives of NCTC 8325 to SEB⁺ and the location of the genes governing SEB could not be determined. Even if they were successful, these experiments would be difficult to evaluate since our laboratory has shown that two determinants (pSN2 and the presumed SEB structural gene) are required for enterotoxigenesis.

Our current understanding of the genetic basis for SEB synthesis can be summed up best as follows: SEB synthesis requires a function (or functions) specified by pSN2, which can exist either as an autonomous replicon or as a chromosomal integrate. The plasmid probably does not contain the structural information for the SEB toxin but instead acts in some fashion critical to the expression of the SEB⁺ phenotype.

GENETICS OF SEA

There have been no reports as yet associating enterotoxin A production with plasmid DNA, as has been shown for SEB. Instead,

SEA appears to be the product of a chromosomal determinant. Our laboratory has shown that SEA production is not plasmid-associated in *S. aureus* and FRI100 (Shafer and Iandolo 1978b) (Table 5). Pattee and Glatz (1980) mapped a determinant critical for SEA production in strain S6, and found that this determinant (*entA'*) resided on the chromosome between the *pur-110* and *ilv-129* markers, in linkage

Table 5. SEA analysis of test strains

Strain	Plasmid Profile	SEA
FRI100	None	+
S6	17.5 Mdal	+
S6 (Cd')	None	+
RN450 (Cd')	Cd' from strain S6	—
RN450	None	—

group III of the *S. aureus* chromosome. In this same study the *entA'* gene of FRI-196E was shown not to be located at this same position between *pur-110* and *ilv-129*. Indeed, the FRI-196E *entA'* determinant could not be located in the three known linkage groups of the *S. aureus* genome. In a second study, Mallonee *et al.* (1981) mapped the *entA'* determinant from 50 SEA⁺ strains, and found that 23 (46%) of these SEA⁺ strains contained the *entA'* determinant in the same location between *pur-110* and *ilv-129* as strain S6. Of the remainder, 22 strains (44%) were genetically incompatible with the multiply-marked auxotrophs these authors used for mapping studies; the remaining 5 SEA⁺ strains tested contained an *entA'* determinant similar to FRI-196E in that these *entA'* determinants were not located within known regions of the staphylococcal genome. This variability in the location of the *entA'* determinant is believed by these authors (P. A. Pattee, pers. comm.) to be presumptive evidence that the *entA'* gene resides on a transposable element.

GENETICS OF OTHER STAPHYLOCOCCAL ENTEROTOXINS

Little has been done to investigate the genetic basis for SEC, SED, and SEE. However, since SEC is closely related immunologically to SEB (Spero *et al.* 1978) and since SEA, SED, and SEE all share common antigenic determinants (Bergdoll 1972) each of these two groups of enterotoxins probably share a common genetic basis.

Betley and Bergdoll (1981) have recently shown that SEC was not associated with extrachromosomal DNA in five strains examined, which is comparable to the finding that the majority of SEB-producing organisms appear not to harbor plasmid DNA associated with enterotoxin production. No information concerning the genetic disposition of SED or SEE or SEF has been presented, to our knowledge.

CONCLUSIONS

It now seems apparent that the previously held notions regarding the genetic regulation of the staphylococcal enterotoxins were in error. Neither plasmid nor chromosomal genes alone can be shown in a straightforward manner to govern the expression of these toxins. Instead the emerging picture is complex and most probably due to the functioning of 2 or more genes. Also because of the clinical and chemical similarities of the enterotoxins, it is very likely that similar regulatory mechanisms will eventually be described for all serotypes.

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ADDENDUM

Since this review was written, a report has appeared (Kahn and Novick. *J. Bacteriol.* 149, 642-649) in which the authors conclude that the plasmid pSN2 is not involved in SEB synthesis. The major experimental proof supporting this position consists of dot hybridizations demonstrating the absence of pSN2 homology in chromosomal SEB producers. We have recently presented evidence (Dyer and Iandolo. 1982. *Absts. of the Ann. Mtg. of the ASM.* abst D43. p. 54.) using the more sensitive Southern blot hybridization refuting Kahn's and Novick's interpretation. Our data demonstrate pSN2 homology in chromosomal SEB producers and in the chromosomal SEB producers we genetically constructed.

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